Role of Heme Oxygenase-1 in the feto-maternal tolerance

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Zusammenfassung

Die Schwangerschaft ist ein komplexes Phänomen, bei dem es zu einer Interaktion zwischen dem mütterlichen Immunsystem und dem Fetus kommt. An der feto-maternalen Grenze kommt es zur Auslösung einer inflammatorischen Reaktion, die für eine normale Implantation und Schwangerschaft notwendig ist. Allerdings kann eine exzessive Entzündungsreaktion zu Schwangerschaftskomplikationen wie dem immunologisch vermittelten Sponatanabort führen. Das zytoprotektive Enzym Hämoxygenase-1 (HO-1) spielt eine sehr wichtige Rolle bei der Kontrolle inflammatorischer Reaktionen. Inwiefern HO-1 für das Gelingen und Bestehen einer Schwangerschaft unabdingbar ist, wurde bisher nicht untersucht. Unsere Hypothese ist, dass HO-1 eine bedeutsame Rolle während der Schwangerschaft spielt. Die Beantwortung dieser wichtigen Frage ist deshalb Hauptziel dieser Dissertation.

Es konnte gezeigt werden, dass eine spezifische Hochregulation des HO-1 Moleküls mittels Gentherapie in einem Mausmodell für Spontanabort zur signifikanten Reduktion der Abortrate führte. Dieser protektive Effekt war mit einer erhöhten Th2/Th1 Zytokinen-Ratio und mit verminderter Apoptose assoziiert.

Ein weiteres Teilziel dieser Arbeit bestand darin, die Rolle des HO-1 Moleküls während der Plazentation zu untersuchen. Dafür wurde eine Trophoblastenstammzelllinie benutzt, die in der Lage ist, zu Riesenzellen zu differenzieren. Die mittels Zinkprotoporphyrin (ZnPPIX) induzierte Expressionssuppression von HO-1 führte zur Verminderung der Überlebensrate von Trophoblastenstammzellen und zur Hemmung von deren Ausdifferenzierung in Trophoblastenriesenzellen.

Um die Rolle des HO-1 Moleküls in anderen Schwangerschaftsprozessen zu untersuchen, wurden Hämoxygenase-1 defiziente (*Hmox1-/-*) Mäuse benutzt. Da die Verpaarung von *Hmox1-/-* Mäuse zu keinem erfolgreichen Abkömmling führt, war ein weiteres Teilziel dieser Arbeit gewesen, den zu Grunde liegenden Mechanismus aufzuklären. Es zeigte sich, dass *Hmox1-/-* Weibchen im Vergleich zu den *Hmox1+/+* Weibchen weniger Oozyten produzieren. Auch konnten die *Hmox1-/-* Oozyten weniger erfolgreich als die *Hmox1+/+* Oozyten fertiliziert werden. Verschiedene Verpaarungsexperimente mit *Hmox1+/+*, *Hmox1+/-* und *Hmox1-/-* Mäusen ergaben einen indirekt proportionalen Zusammenhang zwischen HO-1 Expression und Aborthäufigkeit.

Die hier gewonnenen Daten deuten daraufhin, dass HO-1 eine entscheidene Rolle in der Schwangerschaft spielt. Die gewonnenen Erkenntnisse tragen zum Verständnis der Pathologie

des immunologisch vermittelten Spontaborts bei und können darüber hinaus helfen neue Behandlungsstrategien gegen diese gefürchtete Schwangerschaftskomplikation zu entwickeln.

Schlagwörter

Häm Oxygenase-1, Schwangerschaft, Toleranz, Reproduktionsimmunologie

Abstract

Mammalian pregnancy is a parabiotic union of two genetically different individuals, the fetus and the mother. At the feto-maternal interface, inflammatory processes can occur due to an immune reaction against alloantigens. It is known that some degree of systemic or uterine inflammation is necessary for both normal implantation and pregnancy. However, if this inflammation becomes too excessive it can cause pregnancy complications such as abortion. Heme oxygenase-1 (HO-1), the enzyme responsible for the degradation of free heme, plays a key role in inflammatory processes. Viewing pregnancy mainly as an inflammatory process had led us to the idea that HO-1 may play an important role in pregnancy. Therefore, the main aim of this work was to analyze the role of HO-1 in the different processes related to pregnancy by means of functional studies employing *in vivo* as well as *in vitro* models.

First, we could show that a specific up-regulation of HO-1 in abortion-prone animals by means of an adenoviral vector is able to reduce the abortion rate. This HO-1 up-regulation improved pregnancy outcome by up-regulating the Th2/Th1 cytokines ratio and protecting tissues from apoptosis, suggesting an important role of HO-1 in pregnancy.

In a second part of the work, we aimed to analyze the role of HO-1 in placentation. For that, a trophoblast stem cell line capable of differentiate into trophoblast giant cells was used. Interestingly, a down-regulation of HO-1 by means of ZnPPIX led to diminished survival of the trophoblast stem cells. Furthermore, these cells were unable to differentiate into trophoblast giant cells in the absence of HO-1, strongly suggesting a crucial role of HO-1 in placentation.

Finally, a closer look into the role of HO-1 in pregnancy was performed by using heme oxygenase-1 deficient mice (*Hmox1-/-* mice). Interestingly, *Hmox1-/-* females produce much less oocytes than wild type females. Analyses of the ovaries of both types of females showed differences in follicle development. Furthermore, when fertilized *in vitro*, a significant diminution in the fertilization rate of *Hmox1-/-* oocytes when compared to *Hmox1+/+* oocytes was found. Since the mating of *Hmox1-/-* mice does not yield progeny, we also aimed to clarify whether this is due to problems in the female, in the male or in both. For this, different mating combinations of mice partially or totally deficient in Hmox1 were performed. The analysis of the pregnancy outcome showed that, the less HO-1 in the combination, the higher the fetal rejection.

In summary, a central role of HO-1 in different processes of reproduction could be demonstrated in this work which helps understanding the mechanisms behind pregnancy success.

Keywords:

Heme oxygenase-1, pregnancy, tolerance, reproductive immunology

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15-PGJ2	15-deoxy-delta(12, 14)-prostaglandin J2
AAV	Adeno-Associated Viral Vector
Ab	Antibody
ABC	Avidin-Biotin Complex
Ac-DEVD-pNA	Acetyl-Asp-Glu-Val-Asp p-nitroanilide
Ad	Adenoviral
ADA	Adosine Deaminase
AEC	3-Amino-9-Ethylcarbazole
APC	Antigen presenting cell
APES	3-aminopropyltriethoxysilane
ApoB	Apolipoprotein B
ATP	Adenosine 5´-triphosphate
Bag-1	Bcl-2-associated anthanogene-1
Bcl-2	B-cell lymphoma 2
BSA	Bovine Serum Albumin
BVR	Biliverdin reductase
CD	Cluster of differentiation
cDNA	Complementary DNA
CFDA-SE	Carboxyfluorescein diacetate succinimidyl ester
CFSE	Carboxyfluorescein succinimidyl ester
CHAPS	[3-[3-Cholamidopropyl)dimethylammonio]-1-propansulfonate]
CO	Carbon monoxide
CoPPIX	Cobalt Protoporphyrin IX
COX	Cyclooxygenase
Ct	Threshold cycle
CTLA-4	Cytotoxic T-lymphocyte-associated antigen-4
Cy	Cyan
DAPI	4,6 diamidino-2-phenylindole
DC	Dendritic cell
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco´s Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate

DTT	1,4-Dithio-DL-threitol
E2	Estrogen
EDTA	Ethyldiamintetracetic acid
EGFP	Enhanced green fluorescent protein
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorting
FAM	6-Carboxyfluorescein
FBS	Fetal bovine serum
FCS	Fetal calf serum
FDG	Fluorescein-di-β-galactosidase
Fe	Iron
Fig	Figure
FITC	Fluoresceinisothiocyanate
Foxp3	Forkhead box P3
G418	Geneticin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCs	Giant cells
GFP	Green fluorescent protein
GITR	Glucocorticoid-induced TNFR-related
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
HBSS	Hank´s Balance Salt Solution
hCG	Human Chorionic Gonadotropin
HE	Hematoxylin-Eosin
HEPES	4-2-Hydroxyethylpiperazine-1-ethanesufonic acid
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
Hmox1	Heme Oxygenase-1
HO	Heme Oxygenase
HRP	Horse Radish Peroxidase
HSP	Heat Shock Protein
HTF	Human Tubal Fluid
iDC	Immature dendritic cell
IDO	Indoleamine-2,3-dioxygenase
IFN	Interferon

IHC	Immunohistochemistry
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
IQR	Interquartile range
ires	Internal ribosome entry segment
IVF	*In vitro* fertilization
LaGeSo	Landesamt für Gesundheit und Soziales Berlin
LH	Luteinizing hormone
LIF	Leukemia inhibitor factor
LTR	Long terminal repeats
MAPK	Mitogen-activated protein kinase
MC	Mononuclear cells
MHC	Major Histocompatibility Complex
MLC	Mixed Leukocyte Culture
MoMuLV	Moloney Murine Leukemia Virus
mRNA	Messenger RNA
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenindinucleotide phosphate
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
N.P.	Normal Pregnancy
Nrp1	Neuropilin-1
O.N.	Overnight
P4	Progesteron
PAGE	Polyacrilamide Gel Electrophoresis
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PerCP	Peridin Chlorophyll Protein
PFA	Paraformaldehyd
PFU	Plaque Forming Units
PG	Prostaglandin
PMA	Phorbol 12-mystirate 13-acetat
pNA	p-nitro aniline
PPARγ	Peroxisome proliferator activated receptor-γ

R.T.	Room Temperature
rm	Recombinant murine
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute Medium
RT	Reverse Transcriptase
S.A.	Spontaneous Abortion
SCID	Severe combined immunodeficiency
SD	Standard Deviation
SDS	Sodiumdodecylsulphate
TAMRA	6-carboxy-tetramethyl-rodhamin
TBE	Tris/Borate/EDTA
TBS	Tris Buffered Saline
TBST	TBS Tween 0.05%
TCR	T cell receptor
TdT	Terminal deoxynucleotidyl transferase
TEMED	N,N,N´,N´-Tetramethyl-ethylene diamine
TGF-β	Tumor growth factor-β
Th	T helper
TNF-α	Tumor necrosis factor-α
Treg	Regulatory T cells
TUNEL	TdT-mediated dUTP Nick End Labeling
uNK	Uterine Natural Killer cells
VEGF	Vascular Endothelial Growth Factor
VLDL	Very low density lipoprotein
ZnPPIX	Zinc Protoporphyrin IX

1 Introduction

Mammalian pregnancy is a complex phenomenon that allows that the maternal immune system supports its "semi-allogeneic fetus" during the gestation period without rejecting it. At the same time, the maternal immune system has to be alert and respond to foreign antigens. Since Medawar in 1953 (Medawar, 1953) postulated that the fetus could be considered as an "antigenic foreign body", many related hypothesis have been proposed in order to explain the paradoxical success of pregnancy from the immunological point of view. Since then, the fetus is usually compared to an "allograft", and it is thought that mechanisms leading to successful pregnancy are comparable to those leading to transplant acceptance.

1.1 Tolerance at the feto-maternal interface

Tolerance can be defined as a state of antigen–specific immunological unresponsiveness (Tsokos *et al.*, 2001), as a result of inactivation or death of antigen-specific lymphocytes, induced by the exposure to certain antigens (Abbas and Lichtmann in: Cellular and Molecular Immunology, 2003). Tolerance to self antigens is a common feature of the adaptive immune system. Tolerance mechanisms are initiated during fetal life in the thymus for T-lymphocytes and in peripheral lymphoid organs for B-cell clones by a mechanism of clonal deletion. These early mechanisms eliminate the autoreactive T- and B-cell clones. The autoreactive clones that escape clonal deletion can be neutralized by mechanisms of peripheral tolerance. One of the proposed mechanisms of peripheral tolerance is clonal anergy, which is a process that incapacitates or disables autoreactive clones, and these clones loss the ability to respond to stimulation with the corresponding antigen (Tsokos *et al.*, 2001). Tolerance to foreign antigens may be induced under certain conditions of antigen exposure, e.g. it can be induced if protein antigens are administered systemically at high doses without adjuvants or by oral administration of certain protein antigens (Abbas and Lichtmann in: Cellular and Molecular Immunology, 2003).

Pregnancy is normally considered as a natural state of tolerance against foreign antigens, but how this tolerance is achieved is still controversial. In 1953, the Brazilian Sir Peter Medawar initiated the modern immunology of reproduction by asking: "how does the pregnant mother contrive to nourish within itself, for many weeks or months, a foetus that is an antigenically foreign body?" (Medawar, 1953). In this article, three theories were proposed in order to explain the lack of an immunological reaction from the mother against the fetus:

1. The anatomical separation of the fetus from mother: according to this theory, it was thought maternal and fetal blood circulations were separated by a barrier impermeable to cells. This theory was proved to be wrong, since it is now known that the fetal-maternal interface is a bi-directional exchange surface. Not only maternal immune cells are present at the feto-maternal interface, but also fetal cells are found in maternal circulation. This phenomenon is called feto-maternal microchimerism, being microchimerism defined as a small nonhost cell population (or DNA quantity) from one individual harboured by another individual (Adams and Nelson, 2006). Many reports indicate that microchimerism persists in mother and child even decades later (Bianchi et al., 1996; Lo et al., 1996; Maloney et al., 1999), and new studies have shown that feto-maternal chimerism occurs even very early in pregnancy (Khosrotehrani et al., 2005; Diploma thesis from Nadja Ahmad), showing that the anatomical separation between fetus and mother consists of a "permeable" barrier.
2. The antigenic immaturity of the fetus: this theory pointed out that the fetus is "antigenically immature", not expressing histocompatibility antigens. This theory collapsed very quickly, since fetal skin dendritic cells that are positive for MHC class I but negative for MHC class II are very potent accessory cells in polyclonal T cells responses (Elbe-Bürger et al., 2000).
3. The immunological inertness of the mother: although this theory is not fully rejected, it still does not explain why immune responses against foreign pathogens are normal in pregnancy. However, this theory gave rise to the concept of active **tolerance** mechanisms against the fetus. Nowadays, it is considered that the mother achieves a state of tolerance against the fetus, still being able to elicit normal immune responses against infections. This was nicely shown by a work from Tafuri et al. where it was demonstrated that maternal T cells are aware of the presence of paternal antigens during pregnancy, where they acquire a transient state of tolerance specific for paternal antigens (Tafuri et al., 1995).

1.2 Biology of pregnancy

Despite the differences between the placentas of humans and mice, the immunological mechanisms leading to a successful pregnancy in both species are similar, which validates the use of a mouse model in order to study the role of different molecules in pregnancy. These similarities encourage one to use animal models to find alternative therapies to avoid pregnancies complications.

Many steps are involved in the development of a successful pregnancy, such us proper ovulation and receptiveness of the uterus, fertilization, implantation, placentation, as well as a proper immune response allowing the acceptance of the developing fetus.

1.2.1 Ovulation

Ovulation (i.e., follicular rupture) is a distinct morphological phenomenon that occurs during the transformation of an ovarian follicle into a corpus luteum. The changes that take place in the tissue are pathophysiological in nature since they require acute disruption of dense layers of collagenous tissues. This local damage includes hemorrhage in the vicinity of the lesion on the surface of the ovary (Espey *et al.*, 2004).

The hormonal regulation of ovulation is regulated by different levels of luteinizing hormone (LH), progesterone (P4) and oestrogen (E2) (red and blue in Fig. 1). In humans, ovulation takes place when LH and E2 expression reach their maximum. In mice, this is also accompanied by an augmentation in P4 levels. A schematic representation of hormonal levels during the menstrual cycle is represented in Fig. 1 (from Wang and Dey, 2006).

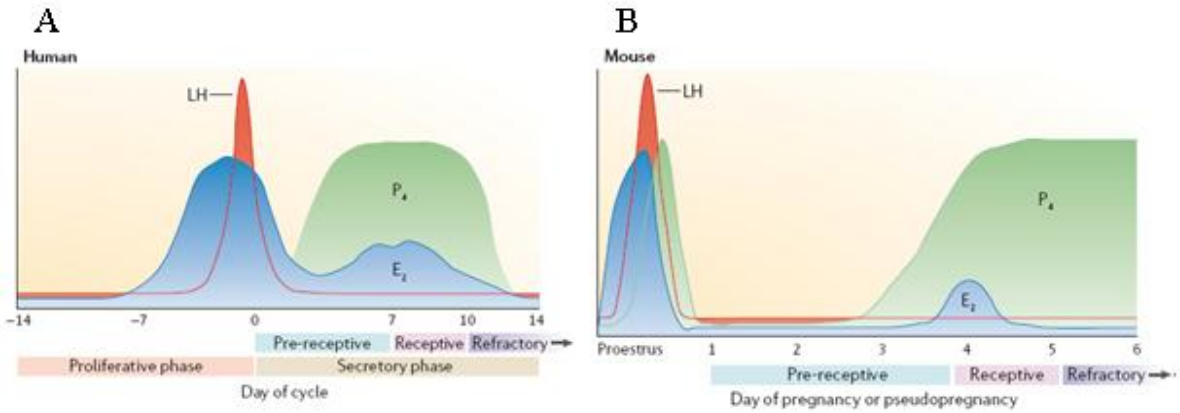


Fig. 1 : Hormonal changes controlling the menstrual cycle in humans (A) and mice (B)

Fig. taken from Wang and Dey, 2006

LH: luteinizing hormone; P_4: progesterone; E_2: oestrogen

Although it is normally assumed that ovulation is a relatively simple phenomenon, it is now evident that the ovulatory process is dependent on the expression of numerous genes, mostly associated with acute inflammatory reactions. These genes include immediate-early genes like tumor necrosis factor-α (TNF-α), cyclooxygenase-2 (COX-2), genes involved in steroido-

genesis, pro-inflammatory genes like interleukin-1β (IL-1β) and IL-6 and genes related to oxidative stress. Concomitant with the inflammatory cascade, there is a cluster of genes that yield protein products to counteract the oxidative stress that is generated in inflamed tissues (reviewed in Espey et al., 2004).

1.2.2 Fertilization and blastocyst formation

The first step in the development of the mammalian embryo involves the fertilization of the oocyte with the sperm. Fertilization is followed by continued cell division, the establishment of cell polarity and compaction to form a morula, followed by a lineage differentiation to form a blastocyst. This period is called pre-implantatory phase and occurs in the oviduct and uterine lumen. A schematic representation of this phase is depicted in Fig. 2 (Wang and Dey, 2006). If the fertilization occurred in a receptive phase, this stage will be followed by the implantatory phase, at which the blastocyst adheres and implants in the endometrium.

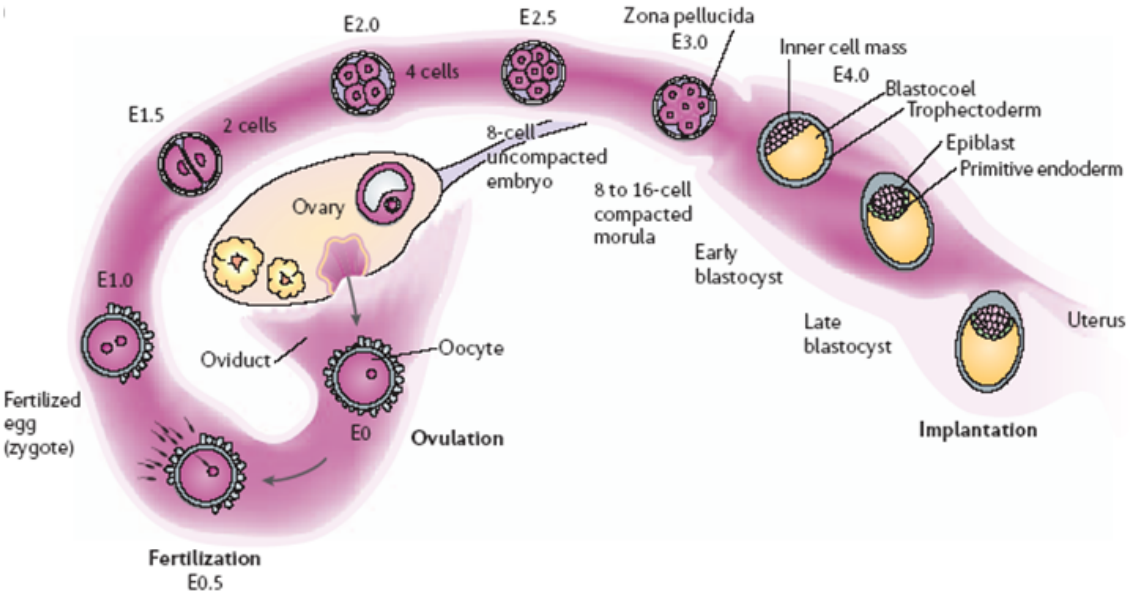


Fig. 2: Preimplantation embryo development in mice

Fig. taken from Wang and Dey, 2006

1.2.3 Implantation

Implantation is the process in which a receptive endometrium allows the attachment of the blastocyst. The independently developing pre-implantation blastocyst becomes then dependent on the maternal environment for its continued development. The main purpose of implantation is to ensure that trophoblast cells firmly anchor into the endometrial stroma, and suc-

cessful implantation is most likely a function of both embryonic and maternal processes (Senturk and Arici in: Immunology of pregnancy). It occurs during a specific period of time called the “implantation window”, that is 96-100 h after fertilization of the ovum in the mouse (Finn and Martin, 1967; Tabibzadeh and Babaknia, 1995), and at between day 19 and day 24 of a normal 28 day cycle in humans (Bergh and Navot, 1992). Females experience physiological changes before implantation takes place: ovulation, copulation and fertilization. These events induce changes in the metabolism as well as in the hormone and cytokine balance, preparing the reproductive tract for the subsequent blastocyst implantation. In order for implantation to be successful, the embryo must have reached a proper stage of development and the endometrium must be receptive (Abrahamsohn and Zorn, 1993). This receptiveness results from adequate exposure of the uterus to progesterone and estrogen (Psychoyos, 1976). During this period, the uterine membrane morphology presents pinopodes, which are large cytoplasmic projections from the uterine epithelium. Although their importance for implantation is unclear, they appear to provide a useful morphological correlate of the receptive state (Sharkey, 1998). Regarding the blastocyst, the loss of the zona pellucida is probably the last event that precedes the beginning of implantation in rodents (Abrahamsohn and Zorn, 1993). The general aspects of the implantation process are similar in humans and rodents, starting with erosion of the uterine epithelium and invasion of the uterine mucosa by trophoblast cells (Arvola and Mattsson, 2001). Mouse trophoblasts penetrate the surface epithelium by displacement penetration, where a number of surface epithelial cells detach from their basement membrane and from each other. These cells degenerate and are then phagocytized by trophoblasts, being the trophoblasts exposed to the bare basement membrane (Tabibzadeh and Babaknia, 1995). When the trophoblasts of the blastocyst contact and attach to endometrial epithelium, it induces the local formation of decidua. The female uterus undergoes changes during this period, process called “decidualization”, a reason why the maternal part at the feto-maternal interface receives the name of decidua during pregnancy. A schematic representation of the implantation process is depicted in Fig. 3 (Wang and Dey, 2006).

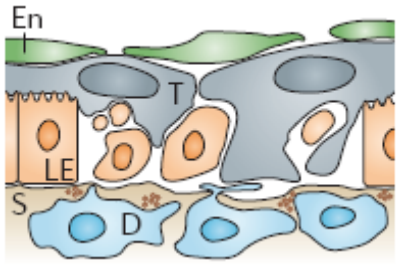


Fig. 3 : Preimplantation embryo development in mice

Fig. taken from Wang and Dey, 2006. T: trophectoderm-derived trophoblast cells; En: blastocyst, represented by the embryonic endoderm; LE: luminal epithelium; S: stroma; D: decidual cells.

1.2.4 Placentation

The implantation period is followed by the formation of the placenta, a process called placentation. The placentas are very different between species, but they all have in common the existence of two separate circulatory systems (maternal and fetal). The placental barrier or interhemal membrane (Benirschke and Kaufmann, 2001) derives from fetal tissues such as the blastocyst wall (trophoblast), which following fusion with the fetal mesenchyme is called *chorion*, together with the *allantois* and the *yolk sac*. All are complemented by the *amnion*, which forms the second fetally derived membranous sac surrounding the embryo. A representative scheme is showed in Fig. 4 (from: Benirschke and Kaufmann, 2001):

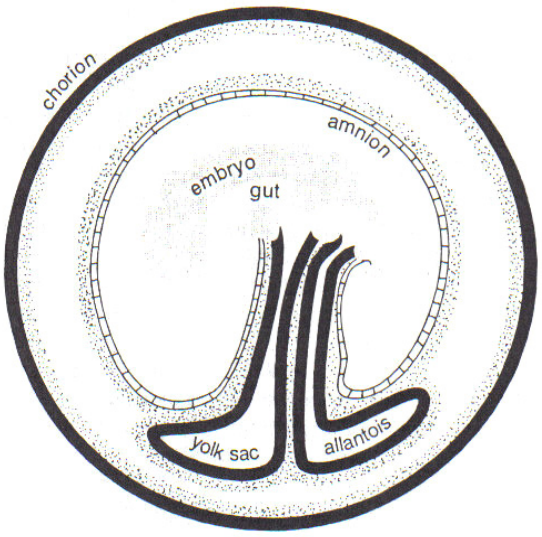


Fig. 4 : Representation of the fetal membranes that contribute to the formation of a placenta

Fig. taken from Benirschke and Kaufmann, 2001

The classification of the different placenta types (Benirschke and Kaufmann, 2001) is defined according to the outer shape, the structure of the feto-maternal barrier, the kind of feto-maternal interdigitation, and the materno-fetal blood flow interrelations. Regarding the placental shape, both human and mouse placentas are a single disk-like zone of intimate feto-maternal contact, being called *discoidal placenta*. This type of placenta possesses the highest degree of feto-maternal interdigitation. Human and mouse placenta have also a similar feto-maternal barrier, which is called *hemochorial placenta*, which has the highest degree of invasion of the trophoblast, that destroy the maternal vessels completely, with the trophoblastic surface directly facing the maternal blood.

However, mouse and human placenta differ in the type of feto-maternal interdigitation and blood flow interrelations. In humans, the placenta is constituted by villi (*villous placenta*), which fit into corresponding endometrial cripts or are directly surrounded by maternal blood. Mice placentas have the most effective kind of interdigitation, the *labyrinthine placenta*, where the trophoblast is penetrated by web-like channels that are filled with maternal blood or fetal capillaries. Here, the blood flow interaction is *countercurrent*, being maternal and fetal capillaries arranged in parallel but with flow in different directions, facilitating passive diffusion. In humans, the blood flow interrelation is called *multivillous flow*.

A schematic representation of human feto-maternal interface is shown in Fig. 5 (from: Arvola and Mattsson, 2001). The tree-like structure of the chorionic villi constitutes the part of the human placenta at which the exchange of nutrients and waste occurs. The yolk sac can be observed between the amnion and the chorion in early pregnancy. By the end of the third month of the human pregnancy, the amnion and chorion have fused (Arvola and Mattsson, 2001).

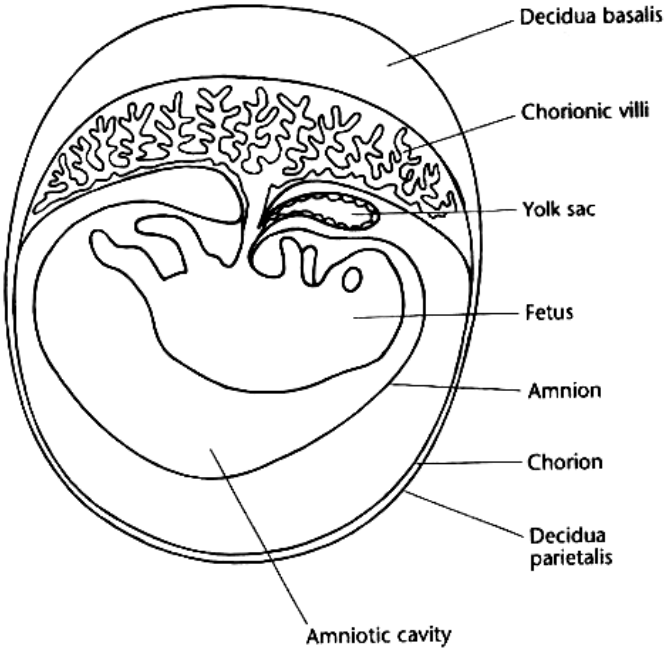


Fig. 5 : schematic drawing of the human placenta and organization of the fetal membranes

Fig. taken from Arvola and Mattsson, 2001

A schematic representation of the mouse placenta is depicted in Fig. 6. Here, the labyrinthine trophoblast constitutes the area of maternofetal interdigitation. In contrast to the human fetus, the mouse fetus is enclosed by the yolk sac (Arvola and Mattsson, 2001).

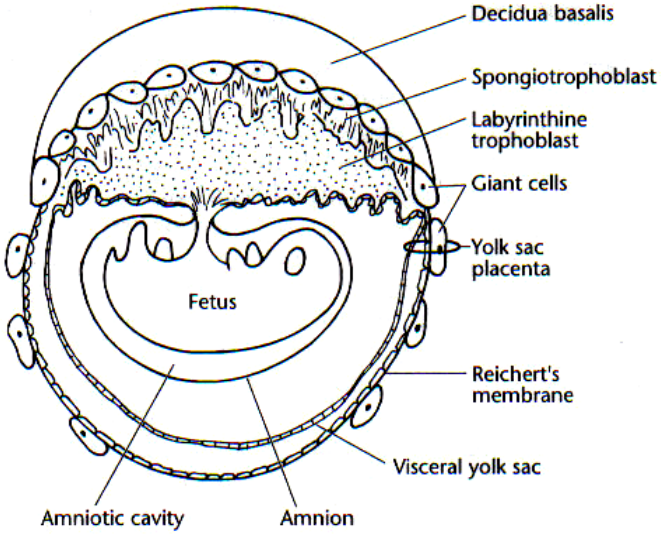


Fig. 6 : schematic representation of the mouse placenta and organization of the fetal membranes

Fig. taken from Arvola and Mattsson, 2001

1.2.5 Immune cells at the feto-maternal interface

Throughout the pregnancy, many immune cells are present at the feto-maternal interface. The dominant lymphocytes during human and murine healthy implantations and later on, are pregnancy associated uterine natural killer cells (uNK cells) (Ashkar and Croy, 2001). It is thought that these uNK cells are bone marrow-derived leukocytes that migrate in large numbers to the pregnant uterus. After migration, uNK proliferate, differentiate, and accumulate in large numbers in specific areas of the uterus between days 2.5 and 12 of murine pregnancy (Redline, 2000). After day 12, uNK cells undergo extensive apoptosis and are dramatically decreased in number and activation (Linnemeyer and Pollack, 1994; Croy *et al*., 1997). These uNK produce IFN-γ at the feto-maternal interface, which contributes to the initiation of vascular modification, decidual integrity and uNK maturation and senescence (Ashkar *et al.*, 2000). In both mice and rats, uNK contain inducible nitric oxide synthase (iNOS) (Hunt *et al*., 1997).

Macrophages are also present in the uterus during implantation. These cells, together with elevated levels of inflammation-associated cytokines such as IL-1 are associated with early implantation (Hunt, 1989). However, other cytokines and growth factors produced mainly by uterine epithelial cells such as TNF-α, TGF-β and GM-CSF are also necessary for facilitation of implantation. All these facts suggest that a limited inflammatory response seems to be a natural component of successful pregnancies (Hunt *et al.*, 2000).

Dendritic cells are also present at the feto-maternal interface, and are likely to regulate immune responses to both uterine infections and placental trophoblast cells (Liang and Horuzsko, 2003). A model proposed by Juretic *et al.* points out that the presence of extravillous cytotrophoblast cells in human pregnancies promotes the recruitment of immature dendritic cells (iDC) and NK cells at the implantation site. It is postulated that, if the stimulation of iDC at implantation site is governed by a strong Th2 response, the function of DC would be tolerogenic, but if they are stimulated under a strong Th1 response, they would become potent antigen presenting cells (Juretic *et al.*, 2004). However, it is known that Th1 cytokines are needed at the time of implantation, making this theory contradictory.

Regarding T cells, it was found that activated γ/δ TCR positive cells are significantly enriched in the decidua as well as in peripheral blood of healthy pregnant women (Szekeres-Bartho *et al.*, 2001). In abortion-prone mice, it was found that TGF-β-producing γ/δ T cells presented suppressor activity dependent on the presence of soluble signals from fetal trophoblast (Clark *et al.*, 1997). Two populations of γ/δ T cells have been described in the murine decidua: an

early population producing Th1 cytokines, and a Th2/3 cell subset that appears later (Arck *et al*., 1999). It was also suggested that γ/δ T cells suppress the anti-fetal immune response through TGF-β production (Suzuki *et al.*, 1995).

1.2.6 Apoptosis at the feto-maternal interface

Apoptosis is a physiologically active mode of cell death that mediates the "safe" deletion of unwanted cells (Gobe and Harmon, 2005). This suicidal pathway is characterized by membrane blebbing, the appearance of highly condensed chromatin and activation of an endonucleolytic process, which leads to the sequential cleavage of genomic DNA. As a result, cells shrink and condense into small membrane-bound "apoptotic bodies", which are then removed by macrophages (Wu *et al.*, 2001). This removal does not produce pro-inflammatory cytokines; on the contrary, it increases the production of anti-inflammatory cytokines (Sepiashvili *et al.*, 2001).

At the feto-maternal interface, it is known that apoptosis plays a role in placental remodelling (reviewed in Jerzak and Bischof, 2002). However, changes in apoptosis at the feto-maternal interface may lead to failing pregnancies, as shown by decreased expression of Bcl-2 and increased expression of Bax in the decidua of failing human pregnancies (Lea *et al.*, 1999). However, it is impossible to determine in the human system if low levels of Bcl-2 and high levels of Bax are a consequence or a cause of the abortion.

A review by Mor and Abrahams (Mor and Abrahams, 2003) points out that during implantation, there is a constant turnover necessary for the appropriate growth and function of the placenta. Furthermore, during the third trimester of human pregnancies, there is increased placental apoptosis that may be involved in the process of parturition. In pregnancy complications such as pre-eclampsia and fetal growth restriction, it was found that an insufficient trophoblast invasion is accompanied by a greater incidence of placental apoptosis.

In spontaneous abortion, there is no evidence that apoptosis may play a determinant role. In murine failing pregnancies, it was shown that apoptosis is not augmented in failing pregnancies, probably due to higher levels of the anti-apoptotic molecule Bcl-2 at the feto-maternal interface (Bertoja *et al.*, 2005).

1.2.7 Factors determining the success or failure of pregnancy

There are many hypotheses trying to explain the onset of a successful or failing pregnancy in murine models as well as in the human situation. Some of these theories are:

1. *Th1/Th2 balance*: many scientists have given importance to the Th1/Th2 cytokines in the onset of pregnancy. They proposed that an excess of Th1 cytokines would be deleterious for pregnancy outcome, leading to pregnancy complications such as spontaneous abortion or pre-eclampsia. This was based on the fact that failing murine and human pregnancies are associated with high Th1 and low Th2 levels (Raghupathy *et al*., 2000; Zenclussen *et al*., 2001). Lin as well as Wegmann proposed in 1993 (Lin *et al.*, 1993; Wegmann *et al.*, 1993) that the balance of pro-inflammatory Th1 cytokines and anti-inflammatory Th2 cytokines is critical to normal pregnancy. According to this, a higher level of Th2 cytokines locally at the feto-maternal interface from human or mouse is related to normal pregnancy (Wegmann, 1993; Piccinni *et al.*, 1998; Krishnan *et al.*, 1996; Saito, 2000; Zenclussen *et al.*, 2001; Zenclussen *et al.*, 2003). Although a Th1/Th2 balance is very important throughout the whole pregnancy, and this has been confirmed by several groups, this explanation turned out to be not sufficient for the explanation of the success or failure of pregnancy, since IL-4/IL10 genetically deficient mice develop normal pregnancies (Svensson *et al.*, 2002). Accordingly, mice lacking simoultaneously IL-5, IL-19, IL-13 and IL-4 do not present abnormal pregnancies, suggesting that these cytokines are not essential for fetal survival (Fallon *et al.*, 2002). In humans, the Th1/Th2 paradigm has also been questioned, since lymphocytes, monocytes and granulocytes from normal pregnant patients produce more IL-12 than those from spontaneous abortion patients (Zenclussen *et al.*, 2002).
2. *Protective role of asymmetric IgG antibodies*: asymmetric antibodies are non-precipitating antibodies that posses an asymmetric structure, due to a high manose carbohydrate group present in only one of the two Fab regions of the molecule (Margni and Binaghi, 1988). They do not form insoluble complexes with antigen, they do not fix complement, and they do not induce clearance of the specific antigen. When they combine with an antigen, they act in a competitive way when they are mixed with precipitating antibodies of the same specificity (Margni, 1994). It was shown that, during pregnancy, the production of asymmetric IgG is considerable increased, and the IgG is of maternal origin and fixed to the membrane of the placental cells (Malan Borel *et al.*, 1991). It is proposed that the presence of these antibodies protect the fetus from being rejected, as these antibodies are not

able to develop an immune response. It is still a valid theory that is in accordance with data pointing out low levels of IL-6 (known to stimulate the production of symmetric normal antibodies), in normal pregnant patients (Zenclussen *et al*., 2000) and may have a connection with the HLA-G expression by the trophoblast (explained later).

3. *Tryptophan catabolism*: tryptophan deprivation might reduce or inhibit some immune cell responses (Thellin *et al.*, 2000). Indoleamine 2,3-dioxygenase (IDO) is an enzymatic protein that catabolizes tryptophan. IDO is synthesized and secreted by the syncytiotrophoblast, and acts by catalysing tryptophan destruction in maternal immune cells that are localised in the placental area. Although Munn *et al*. first proposed that IDO is essential for the success of pregnancy in the mouse (Munn *et al.*, 1998; Mellor and Munn, 1999), the same authors showed later that mice lacking IDO develop normal pregnancies (Baban *et al.*, 2004), pointing out that IDO might be beneficial but not essential for the development of a normal pregnancy. Furthermore, IDO is not expressed in human placentas until 14 weeks of gestation, 13 weeks after implantation (Kamimura *et al.*, 1991) and in mice until day 8 (Munn *et al*., 1998; unpublished data from our lab).
4. *HLA-G expression by the human trophoblasts*: until the moment, the expression of MHC class II in trophoblasts could not be demonstrated. Human trophoblast cells express one class Ia molecule (HLA-C) and all three class Ib molecules (HLA-E, -F and –G) (Hunt *et al.*, 2005). These "non-classical" molecules are recognized by the mother´s immune system as demostrated by the fact that anti-HLA paternal antibodies are common in pregnant women. It is tempting to speculate that these antibodies are of asymmetric nature, but unfortunately no reports could be found on this aspect. It has been proposed that the expression of HLA-G by trophoblast cells may regulate immune cells, targeting all of the major immune cell subsets and programming them into an immunosuppressive phenotype. Thus HLA-G has been proposed to be essential to immune privilege in pregnancy (Hunt *et al.*, 2005). However, definitive proof that HLA-G is required remains elusive, since *in vivo* experiments are difficult to design (Hunt *et al.*, 2005).
5. *Regulatory T cells*: In both rodent and human systems, there are many evidences that there is a special population of cells with immunoregulatory activity, which are called regulatory T cells (Treg cells). These cells were first described as the population in charge of neutralizing autoimmune reactive cells in the periphery that escaped clonal selection in thymus (Sakaguchi *et al.*, 1995). There are many attempts to characterize this population of cells and to understand the mechanism by which they exert their regulatory action. So

far, there is some evidence that the immune suppression by this subset of cells is dependent on IL-10 and TGF-β. (Hara *et al.*, 2001; Kingsley *et al.*, 2002). One of the markers found in many populations of cells with regulatory activity is CD25. However, it is not an exclusive and stable marker for Treg cells, because recently activated T effector cells also express CD25. Additionally, there are some models, in which $CD4^+CD25^-$ cells also have regulatory function (Wood and Sakaguchi, 2003). Nevertheless, the sorting of CD25-expressing cells is still a useful way of enriching Treg cells of this subset, and there are many evidence that this $CD4^+CD25^+$ population plays a regulatory role both *in vitro* and *in vivo* (Gregori *et al.*, 2001; Chai *et al.*, 2002; Kingsley *et al.*, 2002). There are many other possible candidates as markers for the Treg population, such as Cytotoxic T-lymphocyte antigen-4 (CTLA-4), glucocorticoid-induced tumour necrosis factor (GITR), CD122, CD103 and foxp3 as a possible specific molecular marker (Wood and Sakaguchi, 2003). A novel molecular marker may be also Neuropilin-1 (Bruder *et al*., 2004).

In pregnancy, it was first described by Heikkinen *et al*. in human and by Aluvihare *et al*. in murine pregnancies (Heikkinen *et al*., 2004; Aluvihare *et al*., 2004) that the onset of pregnancy is dependent on the presence of this population of cells. Data from our group showed for the first time that mice developing spontaneous abortion have less number of naturally occuring $CD4^+CD25^+$ Treg cells (Zenclussen *et al.*, 2005). Moreover, in abortion-prone females receiving Treg cells arising from normal pregnant mice, fetal rejection could be completely reverted, confirming a very important role of Tregs during pregnancy (Zenclussen *et al*., 2005b). In murine pregnancies, it is thought that Tregs induce a privileged tolerant microenvironment at the fetal-maternal interface (Zenclussen *et al*., 2006). This microenvironment would be characterized by augmented TGF-β, leukaemia inhibitory factor (LIF), and heme oxygenase-1 (Zenclussen *et al.*, 2006a). In humans, it has been reported that Treg mediate a potent inhibition of CD4+CD25- T cells, and that this effect requires cell to cell contact (Sasaki *et al*., 2004). However, it is not described in the literature whether they induce the expression of any of these molecules at the feto-maternal interface.

6. *Leukemia inhibitory factor (LIF):* LIF is a pleiotropic cytokine of the IL-6 family and has different biological actions in various tissue systems (Hilton and Gough, 1991). Nowadays, there is an accumulation of evidence of the importance of LIF in different stages of reproduction. In a mice model, LIF has been shown to be one of the essential cytokines for implantation, since mice lacking LIF could produce normal embryos, but the embryos

failed to implant. Interestingly, when embryos from LIF-deficient mice were transferred into the uteri of wild-type mice, normal implantation occurred, confirming that LIF produced by endometrium is critical for murine implantation (Stewart *et al.*, 1992). LIF seems also to affect sperm survival rates and motility in the fallopian tube, thus playing also a role even in the fertilization process (Attar *et al.*, 2003). There is also evidence suggesting that LIF plays an important role in human reproduction, and is considered as a possible cause of unexplained infertility and multiple failures in implantation (Hambartsoumian, 1998; Steck *et al.*, 2004).

1.2.8 Spontaneous abortion

Between the possible pregnancy complications that may occur during mammalian gestation, spontaneous abortion is one of the most common. It occurs between the first and third month of pregnancy, and ends with the rejection of the embryo. About 20% of all pregnancies end in spontaneous abortion (Essentials of Clinical Immunology), whereas two-thirds of fetuses are lost even before the woman realizes that she is pregnant, reason why it is thought that the percentage of women suffering spontaneous abortion is even higher.

Spontaneous abortion can be caused by genetic disorders, infections, endocrine abnormalities, or autoimmune states as i.e. antiphospholipid syndrome (Essentials of Clinical Immunology). When the cause of abortion does not respond to anatomical, genetical or endocrinological causes, it is very probable that the cause of abortion is of immunological origin. Thus, when a woman suffers from three or more consecutive spontaneous abortions and all other factors have been discharged, the causes of the losses are thought to be immunological. In these cases, the immune system of the pregnant woman does not adapt to the situation and rejects the growing embryo.

1.2.8.1 The CBA/J x DBA/2J model of spontaneous abortion

In the mouse, a useful model of abortion was described for the first time by Clark and co-workers in 1980 and further characterized by Chaouat and co-workers in 1988. In this model of immunological abortion, the combination of CBA/J females with DBA/2J males leads to spontaneous abortion (Clark *et al.*, 1980; Chaouat *et al.*, 1988). The mentioned mice combination develops between 20 and 30% of abortion, and these immunological rejections occur spontaneously without being caused by any treatment of the pregnant mice. Because CBA/J females bear H2k antigens and DBA/2J males bear H2d antigens, this model employs mice that differ in the antigens of the major histocompatibility complex. Different to the

transplant situation, this does not lead to rejection as it is even thought that an allostimulus is necessary for pregnancy to occur. Interestingly, when mating the same females with a BALB/c male, also bearing H2d antigens like the DBA/2J males, these females undergo normal pregnancies. This fact excludes the possibility that the rejection of the fetus may be due to differences in the major histocompatibility antigens.

1.3 Heme Oxygenase-1 (HO-1)

In 1964, Wise and Drabkin (Wise and Drabkin, 1964) first described an enzymatic reaction that converted heme to biliverdin and carbon monoxide. They described that this reaction required NAD and ATP, and that, interestingly, the enzyme source was the light mitochondrial fraction of hemophagous organ of the dog placenta. This report was followed by reports of Tenhunen and co-workers (Tenhunen *et al.*, 1968; Tenhunen *et al.*, 1969; Tenhunen *et al.*, 1972) who described a bile-pigment producing system located in the microsomal fraction of the rat liver, and they named it "microsomal heme oxygenase". This enzyme was described to be responsible for the cleavage of heme at the α-methene bridge resulting in the formation of biliverdin and CO, being biliverdin rapidly reduced to bilirubin by NADPH-dependent biliverdin reductase. A further characterization of the enzyme (Tenhunen *et al.*, 1969) allowed the description of the enzymatic reaction as being dependent on the presence of molecular oxygen and of NADPH or an operational NADPH-generating system. It was also suggested that cytochrome P-450 would play an essential role in the reaction. Further studies (Maines and Kappas, 1974; Yoshida *et al.*, 1974; Maines and Kappas, 1975) led to the identification of the enzyme as a distinct microsomal entity in which its activity does not require cytochrome P-450.

Nowadays it is widely know that Heme Oxygenase (HO), encoded by the *Hmox1* gene, is the enzyme catalyzing the first and rate limiting step in the degradation of heme, to yield equimolecular quantities of biliverdin, CO, and free iron. Biliverdin is then converted to bilirubin via the action of biliverdin reductase, and free iron is sequestered into ferritin, reaction that is schematised in Fig. 7 (Ryter *et al.*, 2002).

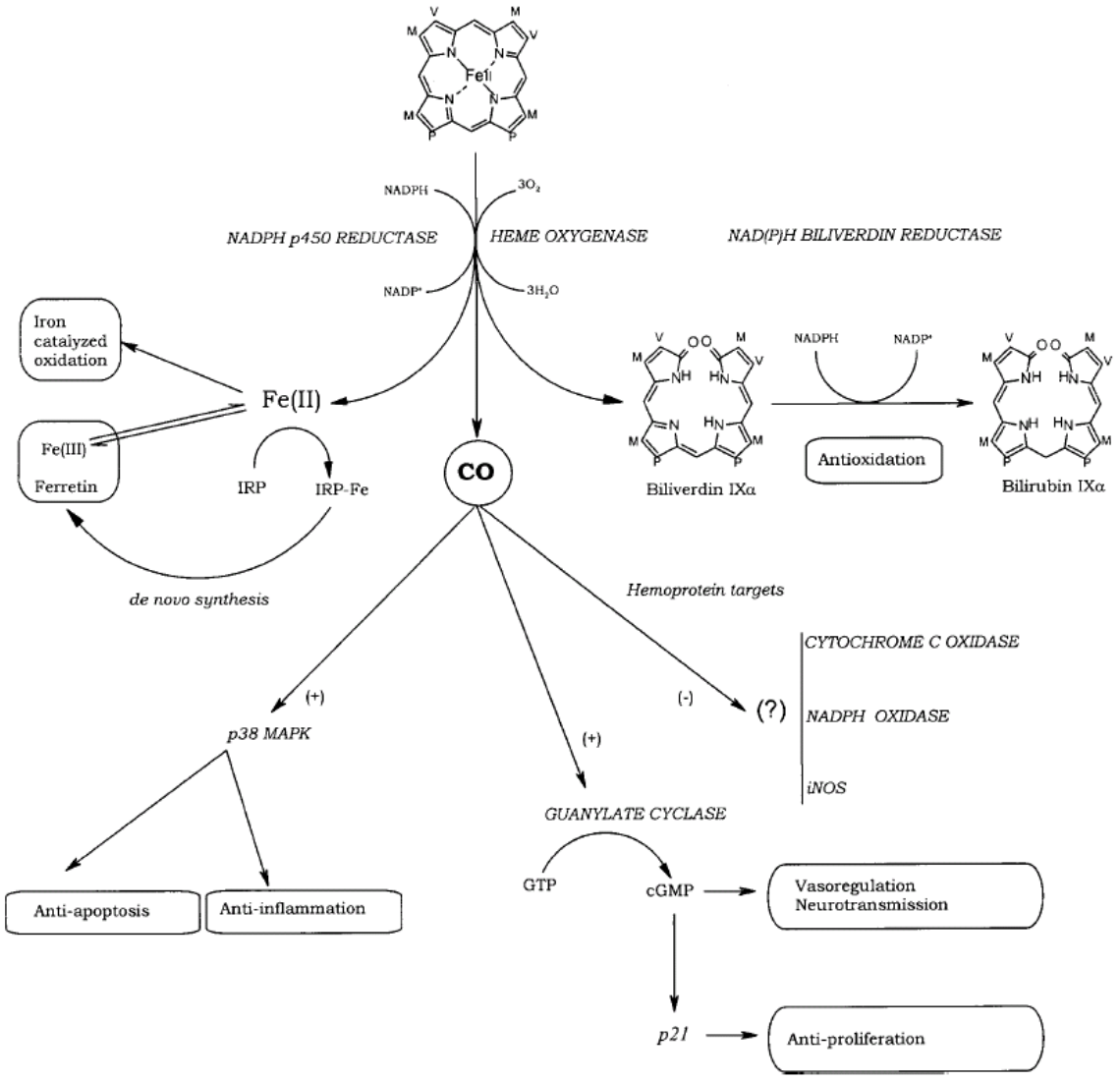


Fig. 7 : Scheme of the reaction of heme oxygenase-1

Fig. taken from Ryter et al., 2002

To date, three different mammalian isoforms have been identified: HO-1, HO-2 and HO-3 (Montellano, 2000; Otterbein and Choi, 2000; Morse and Choi, 2002). HO-1, also known as heat-shock protein (HSP) 32, is very sensitive to several stimuli and agents that cause oxidative stress and pathological conditions, such as heat shock, ischemia, radiation, hypoxia, hyperoxia (Maines, 1997), cytokines (IL-1, IL-6, or TNF-α), heavy metals and nitric oxide. The HO-2 isoform (Maines *et al.*, 1986; Trakshel *et al.*, 1986) is normally referred as constitutively expressed, and is not inducible by the agents capable of inducing HO-1. The list of inducers of HO-2 is limited and include developmental factors, adrenal glucocorticoids, opiates and possibly nitric oxide (reviewed in Maines and Panahian in: Hypoxia: from genes to the

bedside). In human pregnancy, different levels of HO-2 were found between placentas of normal pregnant or abortion patients (Zenclussen *et al.*, 2003b) as well as in patients with pre-eclampsia and fetal growth restriction (Barber *et al.*, 2001), suggesting HO-2 may be regulable in human placenta. The third isozyme, HO-3, was isolated from rat tissues and it presents 90% of aminoacid homology with HO-2 (McCoubrey *et al.*, 1997) but acts as a less efficient heme catalyst (Morse and Choi, 2002).

1.3.1 Tissue distribution and sub-cellular localization

Heme oxygenase is widely expressed in different tissues in mammals, as well as in various species, including unicellular organisms (Maines in: Heme Oxygenase, Clinical Applications and Functions, 1992). Several reports indicate that at least in the rat, HO-1 specific activity is being the highest in the spleen, followed by bone marrow, liver, brain, kidney, and lung, in decreasing order (Tenhunen *et al.*, 1969). In most other tissues not directly involved in erythrocyte turnover or hemoglobin metabolism, low detectable levels of HO-1 are detectable under basal conditions but responds to rapid transcriptional activation by diverse chemical and physical stimuli (Ryter *et al.*, 2006). HO-2 highest activity occurs in the testes, being also expressed in brain and central nervous system, vasculature, liver, kidney and gut (Maines in: Heme Oxygenase: Clinical Applications and Functions, 1992; Maines, 1986; Maines, 1997).

In cells, HO-1 enzymes have been characterized as endoplasmic reticulum (ER)-associated proteins due to the abundant detection of HO-1 activity in microsomal (104,000 g) fractions (Ryter *et al.*, 2006). Both HO-1 and HO-2 contain a COOH-terminal hydrophobic domain that suggests a general membrane compartmentalization (Shibahara *et al.*, 1985). Other reports have also described the presence of HO-1 in mitochondria (Converso *et al.*, 2006; Slebos *et al*., 2007) and in caveolae (Kim *et al*., 2004). However, recent reports showed that HO-1 can also localize to the nucleus and activate transcription factors important in oxidative stress (Lin *et al.*, 2007), and that this form of HO-1 seems to lack the C terminus (Lin *et al.*, 2007).

1.3.2 Relationship between the HO and the NO system

There is some similarity between the systems that generate the gaseous heme ligands carbon monoxide (CO) and nitric oxide (NO). Both the HO and the nitric oxide synthase (NOS) systems have inducible and constitutive forms. The inducible form of the synthase, iNOS, is responsive to some of the stimuli activating HO-1, such as bacterial endotoxins, cytokines, and reactive oxygen intermediates (Nathan, 1992; North *et al.*, 1996). HO-1 and iNOS are highly

induced in inflammatory cells, neutrophils, polymorphonuclear and mononuclear cells (Nath *et al.*, 1992; Willis *et al.*, 1996), whereas HO-2 and neuronal and endothelial forms of NOS are similar in the sense that both are regulated by adrenal glucocorticoids (Weber *et al.*, 1994; Maines *et al.*, 1996), being HO-2 up-regulated and NOS down-regulated (Weber *et al.*, 1994). CO and NO share affinity for the heme molecule, but, unlike NO, which is a free radical and can react with oxygen-derived radicals, CO is not a free radical and cannot produce tissue damage associated with inflammation and cytotoxicity to invading organisms that is caused by free radical species (Maines, 1997).

Induction of HO-1 is likely to modulate NO production, whereas NO has been shown to both inhibit and activate HO activity depending on the conditions (reviewed in Maines, 1997). The proposed regulatory interactions between the HO and the NO system are shown in Fig. 8 (from Maines, 1997).

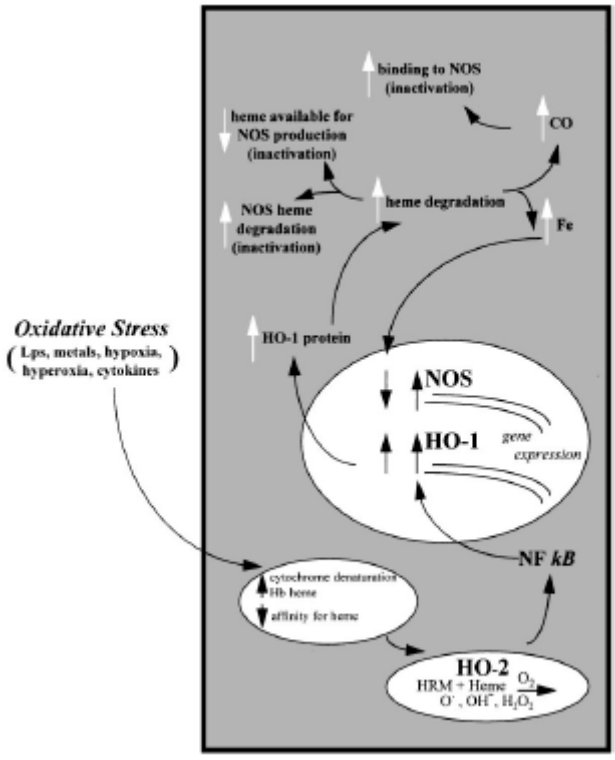


Fig. 8 : schematic representation between the HO and the NOS systems

Fig. adopted from Maines, 1997.

1.3.3 Beneficial effects of HO-1 in different fields of medicine

The most studied isoform of the Heme Oxygenase is the HO-1, a 32 kDa inducible isoform, being its beneficial effects described for many diseases such as atherosclerosis, pulmonary, cardiovascular and renal diseases, as well as in organ transplantation (Morse and Choi, 2002).

The activity of this enzyme is of great importance in cells to protect themselves against oxidative injury. Additionally, HO has been shown to have anti-inflammatory, anti-apoptotic and anti-proliferative effects.

There are many possible explanations to account for the beneficial effects of HO-1. One of them, proposed by Stocker in 1990, suggests that an augmentation of HO-1 represent an antioxidant defence operating at two different stages simultaneously:

1. it decreases the levels of potential pro-oxidants such as heme and heme proteins
2. it increases the tissue concentration of antioxidatively active bile pigments

As it can be seen in Fig. 9 (from Stocker, 1990), the heme molecule serves as a prostetic group of various hemoproteins such as those that transport oxygen or electrons, activate oxygen, or degrade peroxides, which favours a pro-oxidant state. Besides, free heme is capable of catalyzing oxygen radical reactions and is therefore a potent pro-oxidant (Stocker, 1990). Free heme not only stimulates lipid peroxidation (Tappel, 1953), it also causes oxidative damage to DNA and protein (Aft and Müller, 1983, Aft and Müller, 1984) and is cytotoxic to various cell types (Linn and Everse, 1987). Moreover, the iron in the core of the heme structure becomes available to participate in the generation of free radicals (Soares *et al.*, 2001).

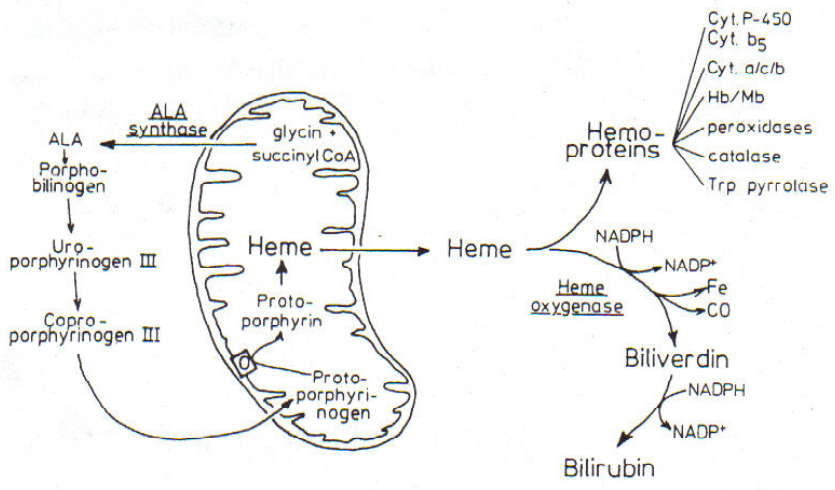


Fig. 9 : Pathway of heme metabolism

Fig. taken from Stocker, 1990.

It has been shown that an increase in HO activity is generally accompanied by a decrease in the cellular concentrations of total and free heme (Maines and Kappas, 1974; Granick, 1975; Maines, 1984) and the lowering of this potent pro-oxidant may already explain some of the beneficial effects associated with HO-1.

An additional protective effect of the HO-1 reaction is related with the accumulation of bilirubin caused by the rapid conversion of biliverdin (product of the HO-1 reaction) to bilirubin by biliverdin reductase. Bilirubin is considered a physiological important antioxidant and will be explained later.

Besides the fact that HO-1 has beneficial effects by decreasing the heme levels, it is also thought that its protective effects are due to the effect of the three products of the catalyzed reaction (CO, biliverdin, free iron).

Beneficial effects of HO-1 have been described in different fields of medicine, such as transplantation (Soares *et al.*, 1998; Coito *et al*., 2002; Tullius *et al.*, 2002; Braudeau *et al.*, 2004), atherosclerosis (Juan *et al.*, 2001), sepsis (Fujii *et al.*, 2003), autoimmune neuroinflammation (Chora *et al.*, 2007) and infections such as malaria (Pamplona *et al.*, 2007). In pregnancy, works from our group (Sollwedel *et al.*, 2005) as well as work obtained and presented in this thesis clearly show that up-regulation of HO-1 have beneficial effects in pregnancy, at least in an experimental model(Zenclussen ML *et al.*, 2006).

1.3.4 Products of the HO enzymatic reaction and their effects

1.3.4.1 Carbon monoxide (CO)

CO is a gaseous second messenger that arises in biological systems during the oxidative catabolism of heme by HO. The endogenous production of CO was described even before the characterization of the HO enzyme (Wise and Drabkin, 1964; Coburn *et al.*, 1967), although it was considered for many years as a waste product with toxic effects but no specific functions. Nowadays, besides its toxic effects at high doses, it is known to mediate salutary effects when applied at low doses. CO is a signal molecule for the generation of cGMP in biological systems (Maines, 1997), and can regulate vasomotor tone as well as neurotransmission, having a regulatory function that may indeed account for the anti-inflammatory effects of HO-1 (Otterbein and Choi, 2000). CO is also known to inhibit platelet aggregation and endothelin-1 production by endothelial cells (Buelow *et al.*, 2001). Besides, anti-apoptotic and anti-proliferative effects were described for CO (Brouard *et al*., 2000; Pae *et al.*, 2004). Moreover,

CO has been shown to affect several intracellular signalling pathways, including guanylate cyclase and the mitogen-activated protein kinases (MAPK) (Otterbein *et al.*, 2000), and these pathways mediate, in part, the known vasoregulatory, anti-inflammatory, anti-apoptotic and anti-proliferative effects of this gas (Ryter and Otterbein, 2004). Recently, it has been reported that CO alone leads to the generation of reactive oxygen species (ROS) which play a major role as signalling molecules to up-regulate peroxisome proliferator-activated receptor-γ (PPARγ), and that PPARγ accounts for the anti-inflammatory effects of CO *in vitro* and *in vivo* (Bilban *et al.*, 2006).

CO was shown to mimic the effects the HO-1 in many models (Sass *et al.*, 2003; Akamatsu *et al.*, 2004; Chora et al., 2007; Pamplona *et al.*, 2007), even administered during an ongoing disease process (Hegazi *et al.*, 2005). However, the effects of CO are lost in *hmox1*$^{-/-}$ macrophages, suggesting the need for HO-1 up-regulation for the inhibitory effects of exogenous CO (Hegazi *et al.*, 2005). It has been suggested that CO initiates an amplification mechanism that results in the production of another product of HO-1, which is the real mediator of the therapeutic effect (Bach, 2005). Alternatively, CO might mediate all of the beneficial effects, but the amplification steps generated by each of the products of HO-1 degradation are essential to generate enough CO at the site of injury to manifest the those effect (Bach, 2006). An interesting theory was proposed by M. Soares at the 5th International Congress Heme Oxygenase 2007, by which it is postulated that complications that are different in nature and outcomes such as artherosclerosis, autoimmune neuroinflammation or malaria present a common feature: the presence of exacerbated inflammation. Interestingly, he proposed that these complications can be reverted by counteracting the exacerbated inflammation by the application of CO, known to have anti-inflammatory properties. Although this theory is based so far only in experimental models, it could be shown e.g. in the case of malaria, that this carbon monoxide may act not only by up-regulating HO-1, but also by binding to free cell hemoglobin, preventing hemoglobin oxidation and the generation of free heme, a potent oxidant that would trigger the pathogenesis of experimental cerebral malaria (Pamplona *et al*., 2007).

1.3.4.2 Iron (Fe^{2+})

The iron released from heme by HO potentially enters a pool of “labile” or “chelatable” iron, where it may be available for cellular processes that depend on iron (Ryter and Tyrrel, 2000). Normally, the free iron is rapidly sequestered into the protein ferritin, and such sequestration can itself lower the pro-oxidant state of the cell by removing the free iron (Balla *et al*., 1992;

Otterbein and Choi, 2000), avoiding iron-dependent oxidative stress. Ferritin-mediated iron chelation inhibits cell-cycle progression, leukocyte migration and fibroblast and endothelial cell apoptosis (Buellow *et al*., 2001). Ferritin has been implied as a cytoprotective molecule in different *in vitro* models (Balla *et al.*, 1992; Lin and Girotti, 1998), and has been proposed as a contributory mechanism underlying HO-dependent protection (Ryter *et al.*, 2006). *In vivo*, it has been shown that the over-expression of H-ferritin protects rat livers from ischemia/reperfusion injury and prevents hepatocellular damage upon transplantation and that its protective effect is related with inhibition of endothelial cells and hepatocyte apoptosis *in vitro* and *in vivo* (Berberat *et al.*, 2003).

1.3.4.3 Biliverdin and bilirubin

Biliverdin, the first product of HO-catalyzed heme cleavage, is enzymatically reduced by biliverdin reductase (BVR) to produce bilirubin. Under normal conditions, biliverdin and bilirubin are processed for rapid elimination (Ryter *et al.*, 2006). Bilirubin is known as a waste product, but it was also shown to be a potent anti-oxidant (Maines, 1997). It has been seen that both biliverdin and bilirubin can act as potent *in vitro* anti-oxidants with possible physiological implications (Stocker *et al.*, 1987; Stocker and Peterhans, 1989; Neuzil and Stocker, 1993). Biliverdin has been also shown to be anti-inflammatory (Vachharajani *et al.*, 2000), and to induce tolerance to cardiac allografts (Yamashita *et al.*, 2004). In ischemia/reperfusion injury, both biliverdin and bilirubin have been shown to be protective *in vivo* (Nakao et al., 2005; Adin *et al.*, 2005).

1.3.5 Heme oxygenase-1 deficiency

So far, only one human case of total heme oxygenase-1 deficiency was described (Yachie *et al.*, 1999). A schematic representation of the observations done in the case of human *Hmox1* deficiency is summarized on Fig. 10.

Briefly, the male patient showed growth retardation, in addition to fever, rash, hepatomegaly, arthralgia without swelling and generalized lymphadenopathy. He also presented fragmented red blood cells in serum, together with giant platelets and dysmorphic monocytes in peripheral blood, with hematuria and proteinuria constantly present. He showed generalized inflammation, disturbances in the coagulation/fibrinolysis system, nephropathy, vascular endothelial cell injury, and asplenia (reviewed in Koizumi, 2007). The patient unfortunately died at the age of 6, but the discovery of this case was of great importance for understanding the im-

portance of HO-1 in the well functioning of the immune system as well as in the metabolism in general. Interestingly, the mother (who presented a heterozygous mutation for HO-1) presented previously two intrauterine fetal deaths (abortions), suggesting that HO-1 plays an important role also in human pregnancies.

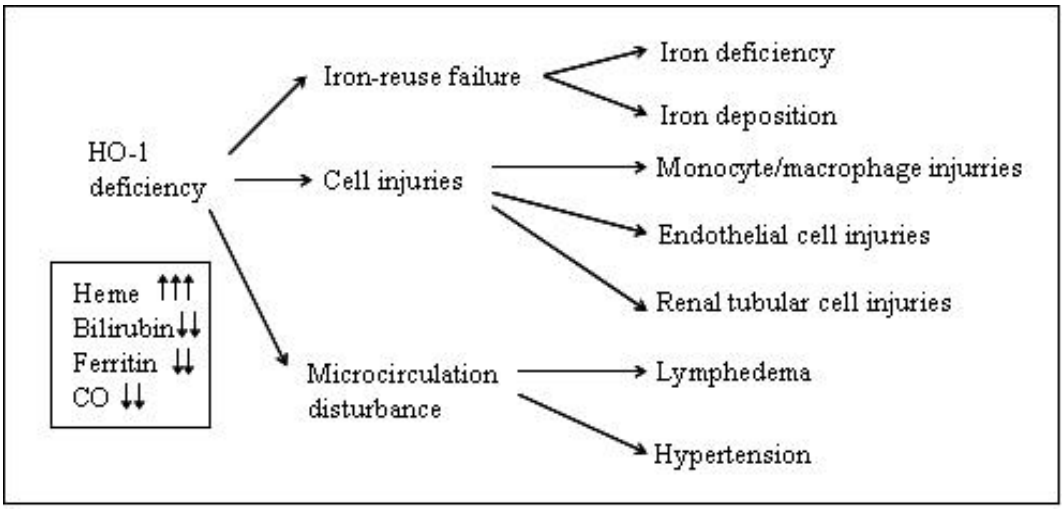


Fig. 10 : Schematic representation of the pathophysiology of *Hmox1* deficiency in humans

Adapted from Koizumi, 2007.

Mice deficient for heme oxygenase-1 (*Hmox1*$^{-/-}$ mice) were first generated and described by Poss and Tonegawa in 1997, in the (129/Sv x C57BL/6) hybrid strain background. These mice were slightly smaller than *Hmox1*$^{+/+}$ or *Hmox1*$^{+/-}$ littermates from birth to early adulthood, but were otherwise indistinguishable (Poss and Tonegawa, 1997). However, after 25 weeks of age they died prematurely or became less active. Besides, after 20 weeks of age *Hmox1*$^{-/-}$ mice presented hypoferremia and anemia with accumulation of tissue iron. These observations led to the authors to propose that these mice have a defect in iron reutilization. Furthermore, these mice presented a progressive chronic inflammatory disease, demonstrated by enlarged spleens and lymph nodes, high splenic and lymph node CD4^{+}:CD8^{+} T-cell ratios with numerous activated CD4^{+} T cells and hepatic inflammatory cell infiltrates (Poss and Tonegawa, 1997). Regarding the maintenance of the colony it was described that matings between heterozygous mice for *Hmox1* did not yield the expected mendelian ratio, being it only 20% of the expected *Hmox1*$^{-/-}$ mice (8% instead of 25%). Even more interesting is the fact that no viable litters were obtained when mating *Hmox1*$^{-/-}$ females and males, suggesting an essential role of HO-1 in pregnancy.

Another colony of *Hmox1* deficient mice was generated by Yet *et al.* in 1999, in a (129Sv x BALB/c) mixed genetic background. Consistent with the data of Poss and Tonegawa, the matings of $Hmox1^{+/-}$ did also not yield the expected mendelian ratio, which led the authors to propose that homozygous mutants present partial embryonic or neonatal lethality (Yet *et al.*, 1999). These mice also present enlarged spleen, but are otherwise macroscopically indistinguishable from $Hmox1^{+/+}$ or $Hmox1^{+/-}$ mice (personal observations).

1.3.6 Role of HO-1 in tolerance

A very interesting paper published in 2005 by Fritz H. Bach (Bach, 2005) points out that many molecules act through a so called "HO-1 amplification funnel". According to this theory, the beneficial effects associated to molecules such as IL-10, rapamycin, 15-PGJ_2, or aspirin, are in fact due to the up-regulation of HO-1 by these molecules. By definition, a given molecule would only function *via* the "amplification funnel" if both of two conditions obtain:

1. the molecule functions only when HO-1 is present and induced, and a product of HO-1 can mediate the functions ascribed to the given molecule in the absence of that other molecule.
2. HO-1 can amplify the therapeutic effects of the other molecule.

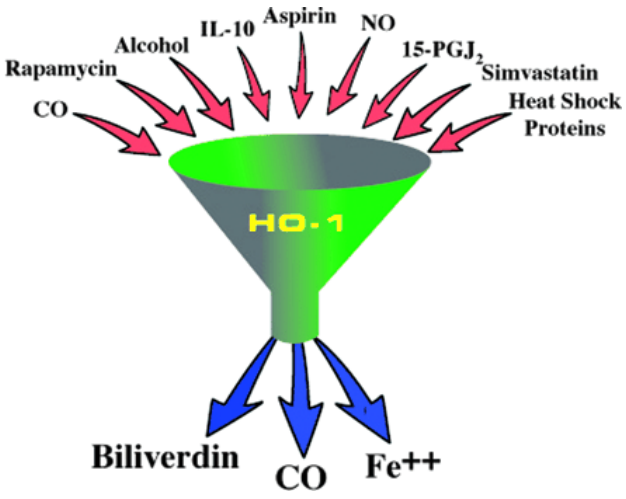


Fig. 11 : HO-1 therapeutic amplification funnel

Fig. taken from Bach, 2005.

There are many molecules that appear to function via the HO-1 funnel, and they are represented in Fig. 11 (Bach, 2005). Although these molecules are not related, they all require the

presence of HO-1 to exert its action, and their actions can be mimicked by HO-1 or its products. The effects obtained in all the cases are related to the achievement of a tolerant state.

1.3.6.1 HO-1 in organ transplantation

Tolerance plays a central role in organ transplantation, and in this regard, HO-1 has been shown to play an important role. Almost all transplanted organs suffer certain degree of ischemia/reperfusion injury, characterized by primary microcirculatory flow disturbances caused by the production of oxygen free radicals and cytokine-mediated inflammatory damage (Katori *et al*., 2002). Besides, after organ transplantation, large amounts of free hemoproteins are released, and it is known that acute exposure to heme is a highly cytotoxic event. In these cases, there are mechanisms to protect the cells against these damages, and in these processes the HO-1 molecule plays a central role. In this regard, many works have already shown that the up-regulation of HO-1 is able to avoid graft rejection. The use of HO-1 inducers such as cobalt protoporphyrin (CoPPIX) has shown beneficial effects through the up-regulation of the HO-1 expression in a steatotic rat liver model of *ex vivo* cold ischemia/reperfusion injury (Amersi *et al*., 1999), and in a mouse model of transplantation of islets of Langerhans (Pileggi *et al.*, 2001). Additionally, in a model where animals tolerated their transplants by means of a treatment with anti-CD40L antibody plus donor-specific transfusion, tolerance could not be achieved in *Hmox1*$^{-/-}$ mice or in mice treated with the HO-1 inhibitor zinc protoporphyrin (ZnPPIX) (Yamashita *et al.*, 2006), suggesting that HO-1 is essential for the tolerance to transplanted organs.

1.3.6.2 HO-1 in pregnancy

Considering the similarities between an allotransplant and a fetus from an antigenically point of view, it is to expect that HO-1 may play an important role in favouring the tolerance against the allogeneic fetus. Although little was known in the last years about the role of HO-1 in pregnancy, many recent studies point out a key role of this enzyme. So far, we could show that mice undergoing abortion presented down regulated levels of HO-1 and HO-2 at the feto maternal interface when compared to normal pregnant mice (Zenclussen *et al.*, 2005). Accordingly, human miscarriage was associated with diminished placental HOs levels (Barber *et al.*, 2001; Zenclussen *et al.*, 2003b). Besides, Kreiser *et al.* reported that the injection of an adenoviral vector containing HO-1 into 15-day pregnant rats lead to increased pup weight (Kreiser *et al.*, 2002). As previously explained, mice lacking HO-1 (*Hmox1*$^{-/-}$ mice) present a variety of disease symptoms, including anemia, abnormal iron-loading, and chronic inflam-

mation, showing *Hmox1*$^{-/-}$ embryos and mice abnormalities like impaired cellular stress response and abnormal responses to endotoxin (Poss and Tonegawa, 1997). Interestingly, *Hmox1*$^{-/-}$ mating pairs did not yield viable litters (Poss and Tonegawa, 1997). These data reflex the fact that no successful pregnancy can be achieved without HO-1, pointing out a key role of this enzyme in pregnancy.

The hypothetical scenario depicted in Fig. 12 (adapted from Zenclussen *et al.*, 2002b) points out that during pregnancy, specially due to the inflammatory nature of blastocyst implantation, there are huge amounts of free heme that need to be degraded.

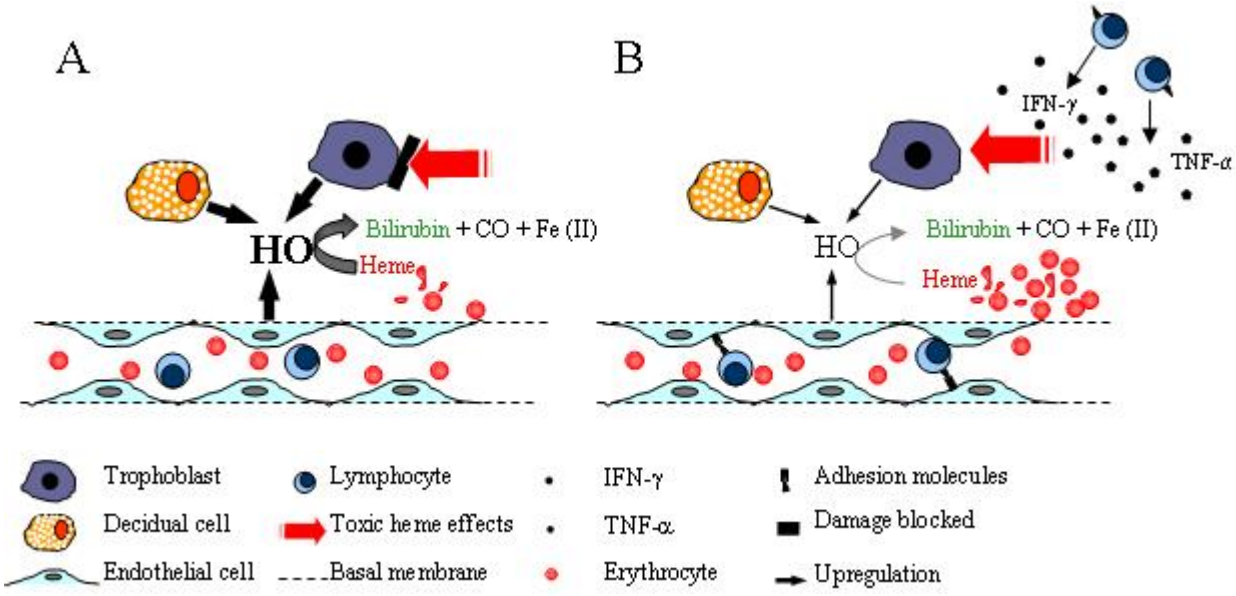


Fig. 12 : Hypothetical scenario of the role of HOs in pregnancy and Th_1-mediated abortion.

Adapted from Zenclussen et al., 2002b.

In a spontaneous abortion situation (B), HO-1 would be expressed but not at sufficient levels, thus being insufficient to degrade the free heme present in the system. The accumulation of free heme, a potent pro-oxidant, would lead to damage of endothelial cells and trophoblasts. In addition, it would enhance the expression of adhesion molecules, which would allow further trafficking of Th1 lymphocytes into the feto-maternal interface, known to be pregnancy-deleterious. In a normal pregnancy situation (A), heme oxygenase would be expressed in sufficient amounts and would be thus able to degrade all the free heme present at the feto-maternal interface, being the previously described cascade avoided.

1.3.7 HO-1 and regulatory T cells (Treg)

Some works point out that there might be a relationship between HO-1 and Tregs. In 2003, Pae found that human $CD4^+CD25^+$ cells from blood constitutively express HO-1, whereas $CD4^+CD25^-$ did not (Pae *et al.*, 2003). Later in 2005, it was demonstrated that HO-1 expression could be induced by Foxp3 gene transfection, and that HO-1 was involved in Foxp3-mediated immune suppression (Choi *et al.*, 2005).

In pregnancy, as already mentioned, a link between Tregs and HO-1 was also found, as mice receiving Tregs to be rescued from spontaneous abortion showed augmented levels of HO-1 at the feto-maternal interface when compared to mice developing spontaneous abortion (Zenclussen *et al.*, 2006). Additionally, the up-regulation of HO-1 by Cobalt Protoporphyrin (CoPPIX) led to augmented levels of mRNA of Neuropilin-1, a suggested marker of Treg (Sollwedel *et al.*, 2005). Interestingly, in a work where tolerance to islet allografts is achieved by inducing HO-1 has shown that if Treg are depleted prior to transplantation, this tolerance is no longer achieved (Lee *et al.*, 2007). All these data suggest a relationship between both systems. However, it has still to be proven that there is in fact a functional important feedback between both systems. Some other authors point out that there is no such relationship between HO-1 and Tregs. For example, the work of Zelenay *et al*. (Zelenay *et al.*, 2007) showed that the frequency of $CD25^+$ or $foxp3^+$ Tregs does not differ between *Hmox1*$^{-/-}$ and *Hmox1*$^{+/+}$ mice, and that Treg isolated from these animals are equally efficient in controlling the proliferation *in vitro* and the expansion *in vivo* of $CD4^+CD25^-$ T cells.

1.4 Gene Therapy

The basic concept of gene therapy involves the introduction of a foreign gene with the purpose of correcting genetic diseases or in order to deliver new therapeutic functions to target cells (Walther and Stein, 1996). However, the use of gene therapy for clinical trials is still controversial and facing many obstacles before the use of DNA as a drug becomes commonplace (Thyagarajan *et al*., 2001). Nevertheless, the use of gene therapy is still of great use in animal models especially in helping to understand the function of certain molecules in different experimental models.

1.4.1 Viral expression systems used in gene therapy

One of the most important aspects of gene therapy is the safe and efficient delivery of DNA, and for that, the choice of the vehicle for delivery. Any ideal vector used as DNA delivery vehicle should have the properties of easy and sustained production and should be immunologically inert. It is desirable that it delivers only in certain cell types and that it can infect both dividing and non dividing cells. An ideal vector should have no size limit regarding the genetic material it can deliver, and in some cases a site-specific integration into the chromosome of the target cell is convenient (Somia and Verma, 2000). There are at the time no ideal vector fullfilling all these properties but it is important that the chosen vector has so many of them as possible.

Although there exist non-viral systems to introduce foreign DNA in tissues or cells, they have as disadvantage an inefficient gene transfer and a transient expression of the foreign gene (Somia and Verma, 2000; Wood and Prior, 2001). Therefore, viral vectors still represent the most suitable vehicles for gene therapy trials.

Viral vectors are obtained by replacing genetic components from the original virus with the therapeutic gene. Depending on the integration of the genetic information into the host genome or not, these vectors are divided in two categories.

- Integrating virus: in this group three main vectors are included: retroviral, lentiviral and adeno-associated viral vectors.
- Non-integrating virus: to this group belongs the adenoviral vector type, which is maintained as an episome.

Some of the viral vectors that are most used in gene therapy trials are described below:

a) Retroviral vectors: these vectors are the most used in gene therapy trials and derive from Moloney murine leukaemia virus (MoMuLV). Retroviral vectors derived from MoMuLV promote the efficient transfer of genes into a variety of cell types, and cause no detectable harm as they enter their target cells. The retroviral nucleic acid becomes integrated into chromosomal DNA, ensuring its long-term persistence and stable transmission to all future progeny of the transduced cell (Boulikas, 1998). Retroviruses have three essential genes which are usually provided separately in packaging cells, in order to make them deficient of replication in the absence of the packaging cell line, being therefore safer. These genes are the gene *gag*, which encodes for viral structural proteins, the gene *pol* encoding for reverse transcriptase/integrase, and the gene *env*, which encodes viral envelope glycoprotein. The *gag* and *pol*

genes are separated from the *env* gene, making the regeneration of a replication competent virus unlikely (Somia and Verma, 2000). A schematic representation of the production of retroviral vectors is schematized in Fig. 13 (Somia and Verma. 2000).

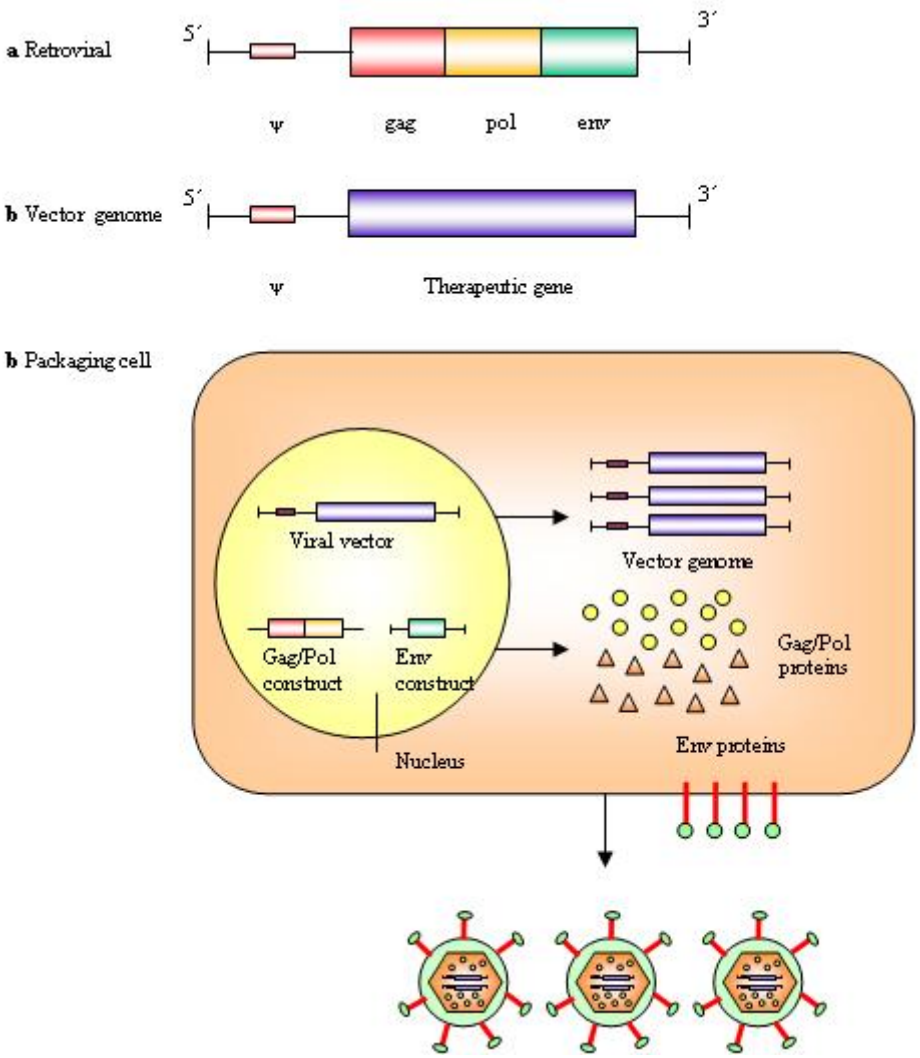


Fig. 13 : Retrovirus-based vectors

Adapted from Somia and Verma, 2000. a) The retroviral genome contains normally the gag, pol and env genes. The Ψ is the packaging sequence and is recognized by the viral proteins for packaging. b) The vector genome, where the gag, pol and env are replaced by the therapeutic gene. c) The packaging cell. The vector genome, by virtue of the Ψ sequence are encapsulated along with the Pol and Gag proteins and assembled under the membrane. The virus buds off from the packaging cell, where it is able to infect other cells but not to replicate.

Other advantage of the retroviral vectors, is that by changing the envelope protein it is possible to change the target cells from ecotropic (infecting only rodent cells) to xenotropic (infecting most mammalian cells except rodent cells), amphotropic (infecting all mammalian cells)

and pantropic (infecting various species) (Danos and Mulligan, 1988; Markowitz *et al*., 1988; Burns *et al*., 1993). One of the disadvantages of these vectors is that they can infect only dividing cells, which constitutes the main limitation of their use. Other mentionable disadvantage is the low viral titers obtained when working with these viruses, and they may be too low to achieve therapeutic levels of gene expression (Boulikas, 1998). Despite these disadvantages of the retroviral vectors, the already mentioned advantages make them one of the most used vectors in gene therapy trials.

b) Lentiviral vectors: they are part of the retrovirus family, and their advantage is that they are able to infect non-dividing cells (Somia and Verma, 2000). One disadvantage that they have is the possibility of recombination and generation of infectious HIV and the non-specific integration in the chromosome. Although there are many trials trying to overcome this disadvantage, their use remains very limited. Additionally, they present the disadvantage of low titer production and decreasing levels of transgene expression over time (Buchschader and Wong-Stall, 2000).

c) Adeno-Associated Viral Vector (AAV): AAV is a small-single stranded DNA virus, non-pathogenic, which requires a helper virus (adenovirus or herpesvirus) for replication and propagation (Somia and Verma, 2000; Mathieu *et al*., 2002). The AAV is capable of integrating into the host genome and can transfer genes in both quiescent and replicative cells (Mathieu *et al*., 2002). The problems related with these vectors are the coding capacity (restricted to 4.5 kb (Somia and Verma, 2000)), and the initial time delay before the beginning of transgene expression (Mathieu *et al*., 2002).

d) Adenoviral vectors: adenoviruses are double-stranded DNA viruses, with a genome flanked by inverted terminal repeats (ITRs), which serve as origins of replication. The wild type adenoviral genome possess 4 major classes of genes: early E1 gene encoding products that activate transcription of the other viral genes; the E2 gene encode proteins that enable DNA replication, while the products of the E3 gene function in counter host cell defence mechanism. Finally, the products of the E4 gene count on the regulation of the viral life cycle (Thyagarajan *et al.*, 2001). The most commonly used replication defective adenoviruses are viruses containing deletions of the E1 and E3 regions, which are also referred as first generation vectors (Graham, 2000). Deletion of E1 renders the adenovirus replication-defective, thus E1 genes are provided in *trans* to allow the production of adenoviruses in cell lines. The E3 gene is normally replaced by the desired (therapeutic) transgene (reviewed in Thyagarajan *et al.*, 2000). Their advantages for the use in gene therapy are that they allow inserts up to 7.5 kb

and that they are produced at high viral titers (Wilson, 1996). Adenoviruses have the ability to transduce dividing and non-dividing cells efficiently, and although they can not integrate into the host genome, they have many advantages that make them very useful in many gene therapy clinical trials, especially in those trials where short-term expression of immunomodulatory molecules can be efficient for the induction of the protective effect, e.g. tolerance. These short-term expression is a consequence of the loss of adenoviral episomes in progeny cells, and the humoral and cellular immune response of the host against the adenoviral antigens, aiming to clear the transduced cells. In adult animals, the transgene is expressed for only a short time, between 5 and 20 days post-infection (Dai *et al.*, 1995). Nevertheless, their ability to transduce dividing and non-dividing cells makes them one of the most used vectors for gene therapy. An overview of the immune response generated after the inoculation of an adenoviral vector is shown in Fig. 14 (from Bangari and Mittal, 2000), in the situation of a first inoculation with an adenoviral vector. Briefly, the first use of an adenoviral vector leads to a strong innate as well as adaptive immune response, resulting in the elimination of transduced cells as well as in the development of neutralizing antibodies. In the case of a high dose of administered vector, a strong innate immune response is obtained, resulting in the proinflammatory cytokines and chemokines that lead to an acute toxic response and hepatotoxicity (reviewed in Bangari and Mittal, 2000).

There are different possibilities to manipulate the immune system through gene therapy. One of them is the *ex vivo* gene transfer of the target molecule e.g. by using adenoviral or retroviral gene transfer, with different therapeutic genes. The advantage of both systems is the local production of the molecule of interest. The disadvantage of the adenoviral gene transfer is that it produces a transient gene expression, being the expression and effects of the molecule of interest limited. Alternatives to overcome this particular disadvantage are offered by retroviral gene transfer, since the integration of the gene of interest to the host genome allows the long term expression of the target molecule. Other possibility is the modification of certain types of cells, e.g. cells of the immune system and they are used as cellular therapy.

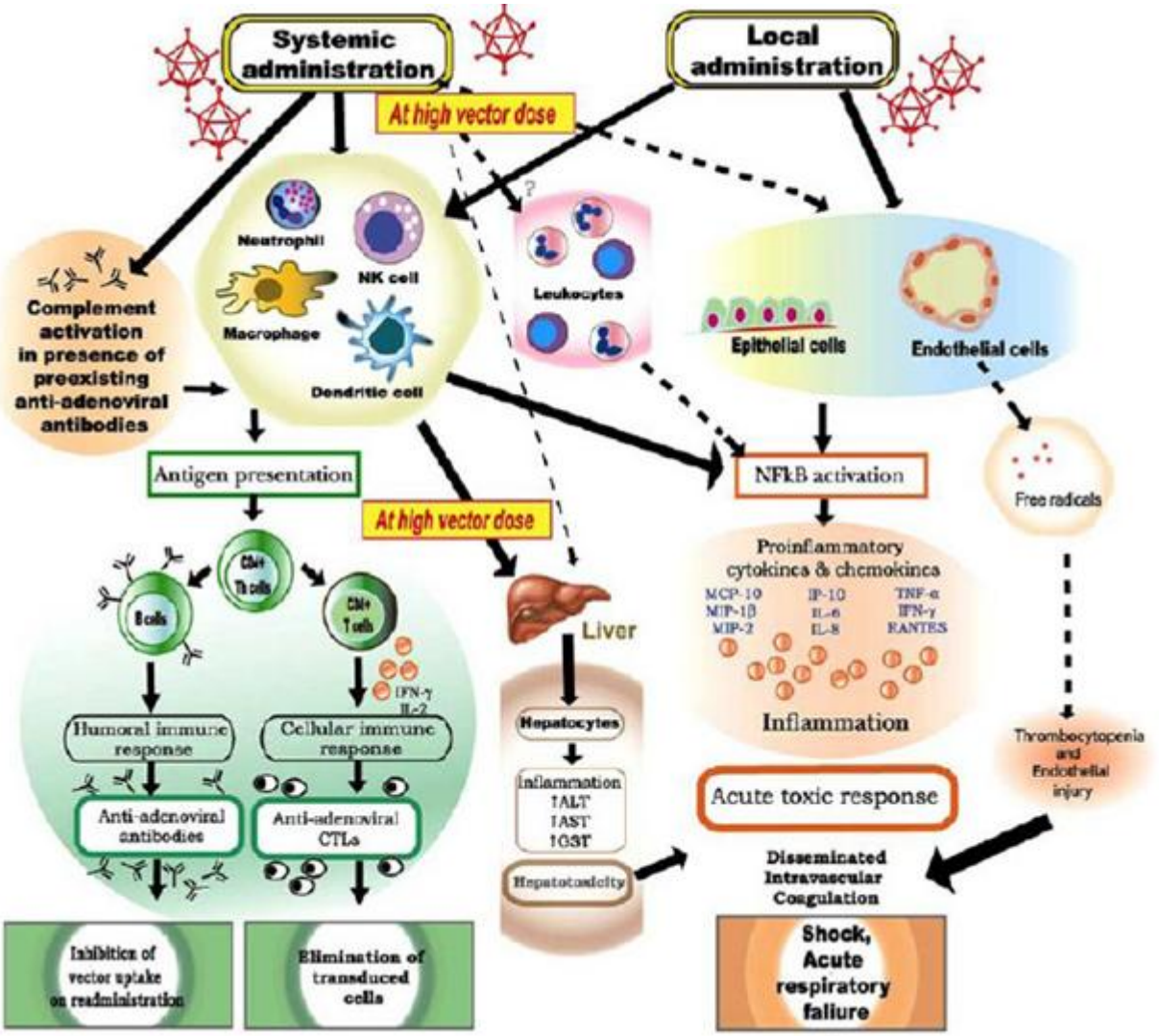


Fig. 14 : Development of adenoviral vector immunity.

Figure taken from Bangari and Mittal, 2000. Briefly, the application of an adenoviral vector leads to innate and adaptive immune responses that ultimately lead to the neutralization of the adenovirus as well as to the elimination of the transduced cells.

One of the most interesting target cells in this regard are T lymphocytes, since they play a key role in immune responses, and they are able to be genetically modified by retroviral vectors. In this case, the retroviral vectors bring the possibility of permanently modify the genome of the T cells, further enabling the selection of the modified cells through a selection marker. This allows the obtainig of a high number of modified T cells that are able to transmit the genetic information to other generation of T cells. Adenoviral vectors have been used so far in many different approaches such as the transfer of factor IX gene in hemophilia B dogs via vein injection (Kay *et al*., 1994) and in mice (Smith *et al.*, 1993), for the transfer of the VLDL

receptor gene for the treatment of familiar hypercholesterolemia in a mouse model (Kozarsky *et al.*, 1996), and for the *ex vivo* transduction of T cells from ADA-deficient patients, between others (Blaese *et al.*, 1995). In humans, trials have been overshadowed by the tragic death of a 18-year-old patient in a Phase I clinical trial in 1998 (reviewed in Somia and Verma, 2000), but nevertheless adenoviral vectors are still widely used in animal models.

The route of administration of the vector determines the efficiency of transgene expression in experimental models. In 1995, Huard *et al.* systematically studied the efficiency of transduction of various adenovirus-recombinants *via* different routes of administration: intramuscular, intracardiac, intraperitoneal, buccal, gastric, rectal, intravenous and nasal. They found out that the route of administration has a major role in determining the transduction efficiency of the different tissues (Huard *et al.* 1995). This should be taken into account when planning experiments tending to obtain a molecule over-expressed in a determined tissue.

1.4.2 Gene transfer to pregnant animals

In reproductive medicine, experimental trials have been made so far only for correcting gene defects *in utero*. The use of gene therapy for improving pregnancy-rate success or avoiding pregnancy-related diseases i.e. miscarriage or pre-eclampsia remains a very distant goal with unresolved moral and ethical aspects. However, gene therapy may be a useful tool in determining the role of genes with a proposed protective role in supporting fetal growth and/or avoiding its rejection experimentally and might further help to identify new targets of intervention. Gene therapy strategies to avoid fetal rejection may include for example the transfer and expression of cytoprotective molecules locally at the fetal-maternal interface. In addition, the *ex-vivo* genetic modification of immune cells and further transfer into pregnant animals for tolerance induction represents a novel and tempting approach.

Interestingly, as reported by Koi *et al.*, the placental "receptivity" to adenovirus depends on the trophoblast differentiation stage (Koi *et al.* 2001). Natural infection of human villous trophoblasts by adenovirus is reduced as the cells differentiate into syncytiotrophoblasts (MacCalman *et al*. 1996, Parry *et al.* 1998). As reported by Senoo *et al.* (2000), the administration of the virus into the amniotic space on 14-days pregnant mice resulted in the expression of the desired gene in the lung, skin surface and epithelium of the digestive tract, while the administration of the recombinant virus into the intra-peritoneal space of the fetus resulted in gene expression in the peritoneum and upper digestive tract. Further, the same authors introduced the adenovirus directly into the systemic circulation of guinea pig fetuses through the umbili-

cal vein and found out that the gene was expressed in multiple organs (Senoo *et al.* 2000), being this route the most effective one for effective fetal somatic therapy. The highest gene expression after gene transfer was found in the liver (80%). Additionally, the administration of recombinant adenovirus *per se* did not have a detrimental effect on the fetuses (Seeno *et al.* 2000). It is also possible that the low lymphocyte infiltration described by these authors (Seeno *et al*., 2000) is due to the immaturity of the fetus or most probably, to the tolerant state at the fetal-maternal interface. Further publications confirm that adenovirus-mediated gene transfer into the fetal systemic circulation resulted in the transgene expression primarily in the liver (Schachtner *et al.* 1999; Themis *et al.* 1999). Although successful, the diffused delivery of vector throughout the fetus, the immune response to the vector and the transfer of genetic material to the mother in this report point out the technical obstacles to be solved if systemic *in utero* gene therapy should became a reality for correcting fetal diseases (Themis *et al.* 1999, Walsh 1999).

Regarding murine placenta, Senoo already reported very low placental transgene expression if the vector was applied into the amniotic space or intra-peritoneally into the fetus on day 14. Accordingly, Laurema *et al.* recently reported that the injection of LacZ-adenovirus into the exocoelomic cavity of rat fetuses led to expression of the gene expression on giant cells while no transduction could be observed in fetuses or rat dams (Laurema *et al.* 2004). These data confirm that the exocoelomic cavity does not offer a route for gene transfer into the fetus. Fetal membranes may act as a barrier, which may naturally prevent adenoviral particles from passing between embryonic cavities (Laurema *et al.* 2004). Interestingly, a work by Okada and co-workers showed that placenta specific gene incorporation can be achieved by lentiviral transduction of murine blastocysts only after the removal of the zona pellucida (Okada *et al*., 2007).

Ex vivo gene transfer

One tempting approach to be used in this emerging field is the transfer of *ex vivo* generated immune cells, which are specific for paternal antigens and carry therapeutic genes as i.e. anti-inflammatory, immunomodulatory or cytokine genes. Experimental protocols from the trans-plantation immunology (Wood and Fry 1999; Hammer *et al.* 2002) suggest that the use of gene-engineered cells may be very effective in pregnancy models since they would act locally at the fetal-maternal interface and may thus prevent inflammatory events, which are known to end in miscarriage. By transferring gene-modified cells into pregnant animals in abortion or pre-eclampsia models (Chaouat *et al.* 1988, Zenclussen *et al.* 2004), gene therapy would be

useful to verify the role of certain genes and to unravel novel pathways involved in pregnancy outcome. Gene therapy approaches in these models could further help designing therapies tending to avoid e.g. immunological rejection of the fetus. Other advantages of this method for using it experimentally in pregnancy models would be the possibility of localization of immunosuppressive/immunoregulatory molecules to targeted sites and the possibility of manipulation of the fetal genotype before implantation takes place since the use of retroviral vectors ensures that the genotype of the target cells in question are permanently modified.

2 Aims of the work

Mammalian pregnancy is a parabiotic union of two genetically different individuals, the fetus and the mother. It is now known that some degree of systemic or uterine inflammation is necessary for both normal implantation and pregnancy, but if this inflammation becomes too excessive it can cause pregnancy complications such as abortion. Heme oxygenase-1 (HO-1), the enzyme responsible for the degradation of free heme, plays a key role in inflammatory processes. Viewing pregnancy mainly as an inflammatory process let us hypothesized that HO-1 may play an important role in pregnancy. Therefore, the main aim of this work was to analyze the role of HO-1 in the different processes related to pregnancy by means of functional studies employing *in vivo* as well as *in vitro* models.

The present work is divided didactically in 4 parts: in vivo up-regulation of HO-1, up-regulation of HO-1 in T cells, effect of HO-1 in the survival and differentiation of trophoblast cells and analysis of the effect of a partial or total loss of Hmox1 on implantation and fertility. The objectives of each part are indicated below:

I. In vivo up-regulation of HO-1

Since it is postulated that a systemic up-regulation of HO-1 is enough to prevent the rejection of allografts, and because the fetus is normally considered as a semi-allogeneic allograft, the aims of this part of the work were:

a) To specifically up-regulate HO-1 in mice undergoing abortion through adenoviral gene transfer.

b) To determine whether this up-regulation improves pregnancy outcome.

c) To investigate possible mechanisms by which HO-1 would exert its action.

II. Up-regulation of HO-1 in T cells

Considering the cytoprotective characteristics of the HO-1 molecule, the aim of this second part of the work was to generate T cells over-expressing HO-1, and to test their potential in the inhibition and modulation of the immune response *in vitro*.

The fulfilment of these aims included:

a) Generation of modified T-lymphocytes over-expressing HO-1 (T_{HO-1}-lymphocytes).

b) Analysis of the function of the generated T_{HO-1}-lymphocytes *in vitro*.

III. Effect of HO-1 on the survival and differentiation of trophoblast cells

The use of the trophoblast stem cell line Rcho-1 helps to elucidate the mechanisms necessary for the differentiation of these cells into giant trophoblast cells, and therefore to understand the mechanisms underlying placentation. The aims of this part of the study were:

a) To analyze the effect of an HO-1 inducer (CoPPIX) or inhibitor (ZnPPIX) on the survival of the trophoblast stem cell line.

b) To analyze the ability of the Rcho-1 cells to differentiate under the influence of CoPPIX or ZnPPIX, in order to determine the role of HO-1 during trophoblast cell differentiation.

IV. Analysis of the effect of partial or total loss of Hmox-1 on implantation and fertility

As animals deficient in *Hmox1* are not able to yield progeny and the mating of mice heterozygous for *Hmox-1* does not yield the expected Mendelian rate, further aims of this work were:

a) To analyze the pregnancy outcome in different combinations of $Hmox1^{+/+}$, $Hmox1^{+/-}$ or $Hmox1^{-/-}$ mice in order to determine whether Hmox1 expression is important at the maternal or paternal side or in both.

b) To determine whether the lack of *Hmox1* has any effect on the fertility of the mice by performing *in vitro* fertilization.

c) To analyze whether *Hmox1* is necessary in the oocytes, in the maternal uterus, or in both, for implantation to occur.

3 Materials and Methods

3.1 Materials

3.1.1 Technical Equipment

Autoclave SL-216/1	WEBECO, Bad Schwartau
Balance	Sartorius, Berlin
Electrophoresis Power supply EPS 3501 XL	Amersham Biosciences, Freiburg
Freezers	New Brunswick Scientific GmbH, Nürtingen
Glas material	Schott, Mainz
ZIEGRA Chip-ice automat	Ziegra Eismachinen GmbH, Isernhagen
Pasteur pipettes	Sarstedt, Nümbrecht
pH-Meter PH 211	Hanna Instruments, Padova, Italy
Refrigerators	Liebherr, Ochsenhausen

3.1.2 Equipment and material for cell culture

15 ml Tubes BD Falcon™	BD Labware, Franklin Lakes, USA
50 ml Tubes BD Falcon ™	BD Labware, Franklin Lakes, USA
Cell culture flasks BD Falcon™	BD Labware, Franklin Lakes, USA
Cell culture plates BD Falcon™	BD Labware, Franklin Lakes, USA
Cell strainers (100 µm pores) BD Falcon™	BD Biosciences Discovery Labware, Bedford, USA
Centrifuge Sigma 3K15	Sigma Laborzentrifugen GmbH, Osterode am Harz
Cryo Tube™ Vials	Nunc, Roskilde, Denmark
Descosept	Dr. Schumacher GmbH, Melsungen
Filters 0,2 µm	Schleicher & Schuelll, Dassel
Filters 0,45 µm	Schleicher & Schuelll, Dassel
Humified incubator	Heraeus, Berlin
Laminair HBB2448	Heraeus, Berlin
Light microscope Olympus CK2	Olympus, Hamburg
Neubauer counting camera	Fein-Optik, Blankenburg

Nitrogen tank MVE Cryosystem 2000	CHART/MVE, Burnsville, USA
S1 Laminar Flow Nuaire	Sarstedt, Nümbrecht
Sterile Pipetes (5,10 and 15 ml) BD Falcon™	BD Labware, Franklin Lakes, USA
Vacuum Filtration Unit	Schleicher & Schuelll, Dassel
Vivaspin 20	Vivascience, Hannover

3.1.3 Equipment and material for Western Blot

Biometra Fastblot	Biometra, Göttingen
Biometra Minigel	Biometra, Göttingen
Chromatography paper Whatman®	Whatman International Ltd, Maidstone, England
ECL™ detection agents	Amersham Biosciences, Buckinghamshires, England
Hypercassette™	Amersham Biosciences, Buckinghamshires, England
Kodak BioMax MR film	Kodak, Cedex, France
Mini Trans-Blot® cell	Bio-Rad Laboratories, Hercules, USA
Mini-PROTEAN® 3 electrophoresis cell	Bio-Rad Laboratories, Hercules, USA
Power supply	Amersham Biosciences, Freiburg
Protec 45 Compact	PMA Bode GmbH, Hamburg
Trans-Blot® Transfer Medium	Bio-Rad Laboratories, Hercules, USA

3.1.4 Equipment and material for flow cytometry

5 ml Polystyrene round-bottom tubes	BD Falcon, Erembodegem, Belgium
Cell strainer, 100 µm Nylon	BD Falcon, Erembodegem, Belgium
BD Plastipak, 1 ml Syringe	BD Falcon, Erembodegem, Belgium
FACS Calibur	Becton Dickinson, Heidelberg, Germany

3.1.5 Equipment and material for molecular biology

ABI PRISM 7700 sequence detection system	Applied Biosystems, Darmstadt
Agilent 2100 bioanalyzer	Agilent Technologies, Waldbronn
Filter tips RNase/DNase free 100 (100 µl)	Greinder BIO-ONE GmbH, Frickenhausen
Filter tips RNase/DNase free 1000 (G) (1000 µl)	Greinder BIO-ONE GmbH, Frickenhausen
Filter Tips RNase/DNase free 10E (10 µl)	Greinder BIO-ONE GmbH, Frickenhausen
HE 99X Max submarine unit	Amersham Biosciences, San Francisco, USA
HP 845x UV-Visible system software	Hewlett Packard, Palo Alto, USA
HP spectrophotometer 8453	Hewlett Packard, Palo Alto, USA
iCycler iQ Real-Time PCR Detection System	Bio-Rad, Munich
Mastercycler personal	Eppendorf, Hamburg
Precision cells made of Quartz SUPRASIL®	Hellma, Müllheim
Safe lock tubes (0.5 ml, 1.5 ml, 2.0 ml)	Eppendorf AG, Hamburg
Thermo-Fast® 96, non skirted PCR plates	ABgene, Epsom, UK
Ultra Clear cap strips	ABgene, Epsom, UK
Ultra-Turrax T25 homogenisator	Janke & Kunkel, IKA Labortechnik, Staufen
Work station	ABgene, Epsom, UK

3.1.6 Equipment and material for immunhistology and immunhistochemistry

ABComplex/HRP	DakoCytomation, Glostrup, Denmark
Accustain® Eosin Y solution aqueous	Sigma-Aldrich Chemie GmbH, Steinheim
AEC+ substrate	DakoCytomation, Glostrup, Denmark
Aquatex®	Merck, Darmstadt
Cover glasses	Menzel-Glaser, Braunschweig
Cryostat HM 500 OM	Microm, Walldorf
Jung tissue freezing medium®	Leica Microsystems Nussloch GmbH, Nussloch
Light microscope Olympus BX40	Olympus, Hamburg
Microscope slides	R. Langenbrinck, Teningen
Microtome HM 400	Microm, Walldorf
Roti®-Histokitt	Roth, Karlsruhe

VECTASHIELD® Mounting medium for fluorescence with DAPI	Vector Laboratories, Burlingame, USA

3.1.7 Equipment and material for *in vitro* fertilization and embryo transfer

Leica MZ75 high performance stereomicroscope	Leica Microsystems GmbH, Wetzlar
Sterile Petri dishes (35 mm)	BD Biosciences, Edembodegem, Belgium
Sterile Petri dishes (60 mm)	BD Biosciences, Edembodegem, Belgium

3.1.8 Chemicals

1,4-Dithio-DL-threitol (DTT)	Fluka Chemie GmbH, Buchs
30% Acrylamid/Bis solution	Bio-Rad, Munich
3-aminopropyltriethoxysilane	Sigma-Aldrich Chemie, Steinheim
[3-[3-Cholamidopropyl)dimethyl ammonio]-1-propansulfonate] (CHAPS)	Fluka Chemie GmBH, Buchs
Agarose 1000	Invitrogen Ltd., Paisley, UK
Ammonium persulfate	Bio-Rad Laboratories, Hercules, USA
Ampicilin	Merck, Darmstadt
AmpliTaq DNA-Polymerase, Buffer + MgCl2	Applied Biosystems, PE, Rodgau-Jügesheim
Aqua ad injectabilia	Delta Pharma, Pfüllingen
Boric Acid	Sigma-Aldrich Chemie GmbH, Steinheim
Bovine serum albumin, Fraktion V (BSA)	Fluka, Buchs, Switzerland
Bromophenol blue	Sigma-Aldrich Chemie, Steinheim
Calcium Chloride	Merck, Darmstadt
Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE)	Molecular Probes, Leiden, The Netherlands
Chloroform	Sigma-Aldrich Chemie GmbH, Steinheim
Citric acid monohydrate	Merck, Darmstadt
Cobalt protoporphyrin chloride (CoPPIX)	Sigma, Steinheim
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich Chemie GmbH, Steinheim
Di-sodium hydrogen phosphate heptahydrate	Merck, Darmstadt
DMEM	Lifetechnologies, Karlsruhe
DNase I RNase free	Stratagene, Amsterdam, The Netherlands

DNase RNase free	Ambion, Huntingdon, UK
dNTPs	Amersham Pharmacia, Uppsala, Sweden
Dulbecco´s Phosphate Buffer Solution (PBS)	PAA Laboratories, Pasching
Ethanol absolut puriss. p.a.	Riedel-de Haën, Seelze
Ethanol	Herbeta Arzneimittel, Berlin
Ethidiumbromide	Carl Roth GmbH, Karlsruhe
Ethyldiamintetracetic acid (EDTA)	Calbiochem, Darmstadt
Fetal bovine serum (FBS)	Biochrom, Berlin
Fetal bovine serum (FBS)	Cambrex Bio Science Verviers, Verviers, Belgium
Fetal calf serum (FCS)	Seromed, Berlin
FicoLite-M	Linaris, Bettingen am Main
Geneticin (G418 Sulfate)	Gibco Invitrogen, Paisley, UK
Glycerol	Serva, Heidelberg
Glycine	Serva, Heidelberg
Guanidine thiocyanate	Fluka Chemie GmbH, Buchs
Hematoxylin solution according to Mayer	Fluka Chemie GmbH, Buchs
Horse serum	Gibco Invitrogen, Paisley, UK
Hydrocloric acid fuming 37%	Merck, Darmstadt
Ionomycin	Sigma-Aldrich Chemie GmbH, Steinheim
L-Glutamine	Sigma-Aldrich Chemie GmbH, Steinheim
Methanol	JT Baker
Mineral oil (mouse embryo tested)	Sigma-Aldrich Chemie GmbH, Steinheim
N,N,N´,N´-Tetramethyl-ethylene diamine (TEMED)	Bio-Rad, Hercules, USA
Oligo DT	Amersham Pharmacia, Freiburg
Paraformaldehyd	Merck, Darmstadt
PCR-Mastermix	Eurogentec, Seraim, Belgium
Penicillin, Streptomycin	Gibco Invitrogen, Paisley, UK
Peracetic acid	Herbeta Arzneimittel, Berlin
Piruvic acid- sodium salt	Sigma-Aldrich Chemie GmbH, Steinheim
Phorbol 12-mystirate 13-acetat (PMA)	Sigma-Aldrich Chemie GmbH, Steinheim
Polybrene	Sigma-Aldrich Chemie GmbH, Steinheim
Poly-L-lisine	Gibco Invitrogen, Paisley, UK
Potasssium dihydrogen phosphate	Merck, Darmstadt
Potassium chloride	Merck, Darmstadt

Potassium hydrogen carbonate	Merck, Darmstadt
Protease inhibitor	Sigma-Aldrich Chemie GmbH, Steinheim
Reaction buffer	Promega, Mannheim
Reverse transcriptase	Promega, Mannheim
RNA-marker	Ambion, Huntingdon, UK
RNase inhibitor	Promega, Mannheim
Saponin	Sigma-Aldrich Chemie GmbH, Steinheim
Skim milk	Fluka Chemie GmbH, Buchs
Sodium azide	Merck, Darmstadt
Sodium carbonate	Sigma-Aldrich Chemie GmbH, Steinheim
Sodium chloride	Merck,Darmstadt
Sodium hydrogen carbonate	Merck, Darmstadt
Sodiumdodecylsulphate (SDS)	Sigma-Aldrich Chemie GmbH, Steinheim
Trizma®-base	Sigma-Aldrich Chemie GmbH, Steinheim
TRIzol® reagent	Invitrogen, Paisley, UK
Trypan blue	Biochrom AG, Berlin
Trypsin-EDTA	Gibco Invitrogen, Paisley, UK
Zinc (II) protophorphyrin IX (ZnPPIX)	Frontier-Scientific, Lancashire, UK
β-mercaptoethanol	Sigma-Aldrich Chemie GmbH, Steinheim

3.1.9 Kits

Annexin-PE apoptosis detection kit I	BD Pharmingen, San Diego, USA
Avidin/Biotin blocking kit	Vector Laboratories, Burlingame, USA
Biorad protein assay	Bio-Rad Laboratories, Munich
CD4+ T cell isolation kit	Miltenyi Biotec, Bergisch Galdbach
$CD4^+CD25^+$ regulatory T cell isolation kit	Miltenyi Biotec, Bergisch Galdbach
Caspase-3 assay kit, colorimetric	Sigma-Aldrich Chemie GmbH, Steinheim
Chariot™	Active Motif, Rixensart, Belgium
In Situ Cell Death Detection kit, POD	Roche Diagnostics GmbH, Penzberg
OptEIA™ Mouse TNF-α ELISA set	BD Pharmingen, San Diego, USA
OptEIA™ Mouse IFN-γ ELISA set	BD Pharmingen, San Diego, USA
OptEIA™ Mouse IL-4 ELISA set	BD Pharmingen, San Diego, USA
OptEIA™ Mouse IL-10 ELISA set	BD Pharmingen, San Diego, USA
peqGOLD Tissue DNA Mini Kit	Peqlab Biotechnologie GmbH, Erlangen

RNase-free DNase Set	Qiagen, Hilden
RNeasy Mini Kit	Qiagen, Hilden
Strata Prep Total RNA Miniprep kit	Stratagene, Amsterdam, The Netherlands

3.1.10 Antibodies for Immunhistochemistry

Anti-Bcl-2 (Clone N-19)	Santa Cruz Biotechnology, Santa Cruz, USA
Anti-goat Ig, biotinylated	Vector Laboratories, Burlingame, UK
Anti-Heme Oxygenase-2	Stressgen, Victoria, Canada
Anti-rabbit Ig, horseradish peroxidase	Santa Cruz Biotechnology, Santa Cruz, USA
Anti-VEGF (Clone P-20), goat plyclonal antibody	Santa Cruz Biotechnology, Santa Cruz, USA
CD3-e (Clone 48-2b) armenian hamster monoclonal IgG	Santa Cruz Biotechnology, Santa Cruz, USA
Goat anti-armenian hamster IgG, biotinylated	Santa Cruz Biotechnology, Santa Cruz, USA
Goat anti-rabbit Ig, biotinylated	Dako, Hamburg
pAb to HO-1 (made in rabbit)	Alexis, San Diego, USA

3.1.11 Antibodies for flow cytometry

Alexa Fluor® 647-Conjugated anti-mouseCD4 (L3T4) monoclonal antibody (Clone RM4-5)	BD Pharmingen, San Diego, CA, USA
FITC anti-mouse CD4 (Clone GK1.5)	BD Pharmingen, San Diego, CA, USA
PE-Cy5 anti-mouse CD8a (Ly-2) (Clone 53-6.7)	BD Pharmingen, San Diego, CA, USA
PE anti-mouse CD25 (Clone PC61)	BD Pharmingen, San Diego, CA, USA
PE anti-mouse IFN-γ	BD Pharmingen, San Diego, CA, USA
PE anti-mouse IL-10	BD Pharmingen, San Diego, CA, USA
PE anti-mouse IL-4	BD Pharmingen, San Diego, CA, USA
PE anti-mouse TNF-α	BD Pharmingen, San Diego, CA, USA
PE-Cy7-conjugated hamster anti-mouse CD69 Monoclonal antibody (Clone H1.2F3)	BD Pharmingen, San Diego, CA, USA
PE-Cy7-conjugated hamster anti-mouse CD95 (Fas) monoclonal antibody (Clone Jo2)	BD Pharmingen, San Diego, CA, USA
PerCP anti-mouse CD3e (145-2C11)	BD Pharmingen, San Diego, CA, USA

3.1.12 Antibodies for cell culture

Anti-mouse CD3ε NA/LE (Clone 145-2C11)	BD Pharmingen, San Diego, CA, USA
Anti-mouse CD28 NA/LE (Clone 37.51)	BD Pharmingen, San Diego, CA, USA
Anti-mouse IFN-γ NA/LE (Clone XMG1.2)	BD Pharmingen, San Diego, CA, USA
Anti-mouse IL-12 (p40/p70) (Clone C17.8)	BD Pharmingen, San Diego, CA, USA

3.1.13 Antibodies for Western Blot

Anti-β-actin	Delta Biolabs, Gilroy, USA
Anti-Heme Oxygenase-1	Stressgen, Victoria, Canada
Anti-GAPDH	Santa Cruz Biotechnology, Santa Cruz, USA
Anti-goat IgG, biotinylated	Vector Laboratories, Burlingame, UK
Anti-rabbit IgG, biotinylated	Vector Laboratories, Burlingame, UK
Anti-rabbit IgG, HRP conjugated	Santa Cruz Biotechnology, Santa Cruz, USA
Rabbit anti-prolactin-like protein A	Chemicon International, Temecula, USA

3.1.14 Membrane dyes

5-(and-6)-CFDA-SE	Molecular Probes, Leiden, Holland

3.1.15 Cell lines

293 cells	American Type Culture Collection (ATCC)
GP+E 86 packaging cell line	Dr. A. Flügel, MPI für Neurobiologie, Abt. Neuroimmunologie, München
NIH 3T3, mouse fibroblasts	DSMZ, Braunschweig
Rcho-1 trophoblast cell line	Dr. Michael Soares, Department of Physiology, University of Kansas Medical Center, Kansas City, USA.

3.1.16 Expression vectors

PLXSN *Amp^R, Neo^R, pBR322ori, Ψ^+,PSV40, 5/3-LTR-Promoter*	Clontech

3.1.17 Hormones

Human chorionic gonadotropin (hCG)	Sigma-Aldrich Chemie GmbH, Steinheim
Pregnant mare serum gonadotropin (PMSG)	Sigma-Aldrich Chemie GmbH, Steinheim

3.1.18 Animals

BALB/c males	Harlan Winkelmann, Borchen or BgVV, Berlin
CBA/J females	Charles River, Les Oncins, France
DBA/2J males	Charles River, Boston, USA

Hmox-1$^{+/+}$*, Hmox-1*$^{+/-}$ *and Hmox-1*$^{-/-}$ females and males were originally generated by Dr. Yet, Harvard Medical School, Boston, USA. The colony of mice used in this work were maintained at the Instituto Gulbenkian de Ciencia, Oeiras, Portugal, at the group of Prof. Miguel Soares. After a MTA agreement with Dr. Yet, animals were kindly provided by Prof. Soares and the colony is being currently maintained in our group.

3.1.19 Buffers and Media

DMEM	Invitrogen, Gibco, Karlsruhe
HBSS	Invitrogen, Gibco, Karlsruhe
RPMI with L-Glutamin	Invitrogen, Gibco, Karlsruhe
Embryomax Human® Tubal Fluid (HTF)	Millipore Corporation, Millerica, MA, USA
M2 Media	Sigma-Aldrich Chemie GmbH, Steinheim

Packaging cell line media:

DMEM with		1 mM	Natriumpiruvat
		2 mM	L-Glutamin
		100 U/ml	Penicillin
		100 µg/ml	Streptomycin
		10%	Fetal Calf serum
	+/-	1 mg/ml	G-418

2 x HBSP Buffer:	50 mM	Hepes
	10 mM	KCl
	12 mM	Dextrose
	280 mM	NaCl
	1,5 mM	Na_2PO_4 x 2 H_2O

T-cell media:

RPMI with	2,05 mM	L-Glutamin
	50 µM	β-mercaptoethanol
	1 mM	Na-Piruvate
	100 µg/ml	Penicillin
	100 U/ml	Streptomycin
	10% (v/v)	Fetal Bovine Serum (Cambrex)

Rcho-1 proliferative cell media:

RPMI with	2,05 mM	L-Glutamin
	50 µM	β-mercaptoethanol
	1 mM	Na-Piruvate
	100 µg/ml	Penicillin
	100 U/ml	Streptomycin
	20% (v/v)	Fetal Bovine Serum (Cambrex)

Rcho-1 differentiation cell media:

RPMI with	2,05 mM	L-Glutamin
	50 µM	β-mercaptoethanol
	1 mM	Na-Piruvate
	100 µg/ml	Penicillin
	100 U/ml	Streptomycin
	10% (v/v)	Horse serum

Solutions for flow cytometry

FACS Buffer:	1%	BSA
	0.1%	NaN_3
	in PBS	
Saponin solution:	0.1% Saponin	
	in PBS	
Lysis buffer	1.5 M	NH_4Cl
	10 mM	$KHCO_3$
	100 mM	EDTA
	in distilled water	

Solutions for magnetic cell isolation

MACS Buffer	0.5% BSA	
	2mM EDTA	
	in PBS, pH 7,4	

Solutions for molecular biology

Solution D:	3.676 g	(tri)-Nacitrat-Dihydrat
	236.32 g	Guanidine-Isothiocyanate
	2.5 g	N-Lauroylsarcosin
	ad 500 ml	DEPC-H_2O, pH 7,0
Loading buffer	34 mg	Bromophenol blue
	3.4 ml	Ficolite-M (δ=1,091)
	10.2 ml	milli Q water
5x TBE	27 g	Trizma®-base
	13.75 g	Boric acid
	10 ml	EDTA 0,5 M pH 8

Solutions for immunohistochemistry

Citrate buffer	1.8 mM	Citric acid
	8.2 mM	Sodium citrate

Solutions for SDS-PAGE and Western Blot

Transfer buffer pH 8,3	25 mM	Tris-Base
(for semi-dry blot)	150 mM	Glicine
	10%	Methanol
	in distilled water	
Transfer buffer pH 8,3	25 mM	Tris-Base
(for wet blot)	150 mM	Glicine
	20%	Methanol
	in distilled water	
Running buffer	25 mM	Tris-Base
	192 mM	Glycine
	0,1%	SDS
	in distilled water	
4x sample buffer	1 ml	0,5 Tris/HCl pH 6,8
	1,6 ml	10% SDS
	3,7 ml	Ficolite-M (□=1,091)
	0,4 ml	□-mercapthoethanol
	0,2 ml	bromophenol blue (1% in ethanol)
	1,2 ml	dist. H_2O
10x TBS	200 mM	Tris-base
	80 g/L	NaCl
	10%	SDS
	pH 7,6 in distilled water	

Stacking gel (5%)	1.3 ml	29% acrylamide, 1% bisacrylamide
	2.5 ml	0,5 M Tris/HCl pH 6,8
	0.1 ml	10 % SDS
	6.1 ml	dist. H_2O
	0.01 ml	TEMED
	0.05 ml	10% ammonium persulphate sol.
Running gel (10%)	3.3 ml	29% acrylamide, 1% bisacrylamide
	2.5 ml	1.5 M Tris/HCl pH 8,8
	0.1 ml	10 % SDS
	4.1 ml	dist. H_2O
	0.005 ml	TEMED
	0.05 ml	10% ammonium persulphate sol.

3.2 Methods

3.2.1 Over-expression of HO-1 through adenoviral gene transfer in a murine model of abortion

3.2.1.1 Recombinant adenoviruses

An adenovirus coding for HO-1 and GFP (AdHO-1/GFP) was constructed using the pAdEasy and pAdTrack-CMV system in 293 cells. AdHO-1/GFP contains two expression cassettes, one for the HO-1 and the other for the GFP, both with the human cytomegalovirus (CMV) promoter, with the human HO-1 cDNA fused to a Flag sequence in its 3´end and a polyA sequence. This adenovirus was designed and produced in INSERM (France) and was a kind gift of Dr. Ignacio Anegon. The adenovirus containing EGFP, which was used as a control, was a kind gift of Dr. Michael Willem (Institute of Neurobiology, Max Planck, Martinsried, Germany). The propagation and purification of both recombinant adenoviruses was performed in 911 cells as previously described (Fallaux *et al.*, 1996; Ritter *et al.*, 1999). In brief, 911 cells were infected at a multiplicity of 5-10 and harvested after 36-48 hrs. The virus was released by five freeze-thaw cycles and purified by two CsCl-gradients (Graham and Prevec, 1991). The banded virus was recovered, desalted over sephadex columns (Pharmacia, Erlangen,

Germany) and kept in virus storage buffer after addition of 10% Glycerol at –80°C. Titration of the virus concentration after elution from the column was performed by plaque assay on 911 cells. The propagation of the adenoviruses as well as the determination of their viral titer was routinely performed by Heinz Tanzmann, member of the Institute of Medical Immunology.

3.2.1.2 Murine abortion-prone model: animals and treatment

All animals were maintained in a barrier animal facility with a 12 h light/dark cycle. Animal care and experimental procedures were followed according to institutional guidelines and conformed to the requirement of the state authority for animal research conduct (LaGeSo Nr. 0062/03 and 0070/03, Berlin). The previously described immunological murine model of abortion was used, in which the mating combination CBA/J x DBA/2J represents the abortion-prone group, being the combination CBA/J x BALB/c the normal pregnancy control. Two months old CBA/J females were mated with 2-4 months old BALB/c or DBA/2J males, checked twice a day for vaginal plugs and separated from the males if pregnant. The day of vaginal plug detection was considered as day 0 of pregnancy. Pregnant females mated with BALB/c were considered as group (1) and received PBS intraperitoneally (i.p.) (n=15). DBA/2J-mated females were then randomized and divided in the following groups:

2) Abortion-prone group + PBS i.p. (n=11)
3) Abortion-prone group + 1.10^5 PFU AdHO-1/GFP in PBS i.p. (n=7)
4) Abortion-prone group + 1.10^5 PFU AdEGFP in PBS i.p. (n=7).
5) Abortion-prone group + 1.10^8 PFU AdHO-1/GFP in PBS i.p. n=8)
6) Abortion-prone group + 1.10^8 PFU AdEGFP (n=7)

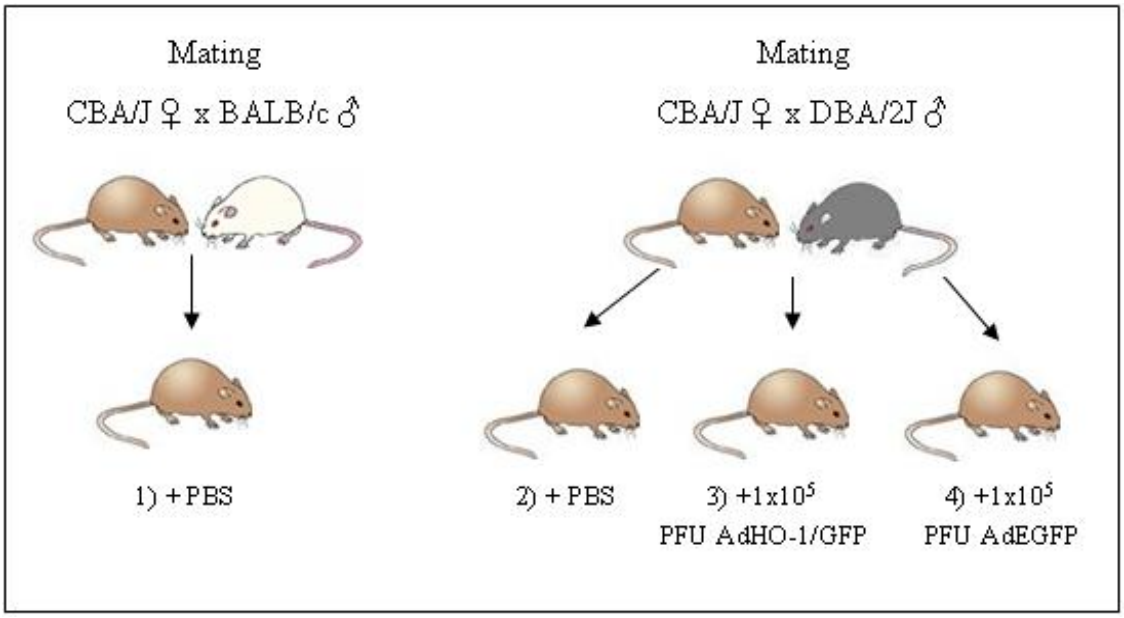


Fig. 15 : Experimental setting.

CBA/J females were mated either with BALB/c males (normal pregnant combination) or with DBA/2J males (abortion-prone combination). The day of the plug detection was considered as day 0 of pregnancy. Injections were made intraperitoneally (i.p.) on day 5 of pregnancy.

All injections were made i.p. on day 5 of pregnancy, which is known to be in the implantation window (Psychoyos, 1986). A schematic representation of the experimental setting is shown in Fig. 15.

The groups 5 and 6 are not included in the schematic representation, since its data will be shown as a table in the appendix. On day 14 of pregnancy, the females were sacrificed, the uteri removed and the implantation sites were documented. The abortion sites were identified by their small size accompanied by a necrotic, haemorrhagic appearance, compared with normal embryos and placentas.

The percentage of abortion was calculated as the ratio of abortion sites and total implantation sites using the following formula:

$$\text{Abortion rate} = \left(\frac{Abortion\,events}{Implantation\,sites} \right) \times 100$$

From each animal, different organs were taken for further analysis. Spleens were kept in cold RPMI for flow cytometry until further use. Two small portions of decidua were snap-frozen

and kept at -80°C for protein and RNA isolation. The rest of the decidua was used for flow cytometry and for that, it was cut in small pieces and kept in HBSS at 4°C until further use. Placentas were snap-frozen and kept at -80° until use (for protein and RNA isolation).

3.2.1.3 Spleen and decidual lymphocytes isolation

Flow cytometry analysis was performed using decidual and spleen lymphocytes. The isolation of mononuclear cells (MC) from decidua was performed as previously described (Zenclussen *et al*., 2003; Zenclussen *et al*., 2004). Briefly, decidual samples were washed with PBS, cut into small pieces, collected in HBSS medium containing 1mM DTT and incubated with agitation for 20 min at 37°C. Thereafter, cells were filtered through a 100 μm net and collected in a fresh tube where they were washed with RPMI medium containing 10% FCS. The procedure was repeated twice, with HBSS without DTT, all supernatants were collected and washed with RPMI medium containing 10% FCS. After that, a Ficolite-M gradient was performed following the instructions of the manufacturer (Linaris, Bettingen am Main, Germany).

Spleen tissues were crushed against a 100 μm net, collected in medium containing 10% FCS and the erythrocytes were lysed with lysis buffer containing NH_4Cl, $KHCO_3$ and EDTA for 10 min at RT. After erythrocyte lysis, cells were washed once with RPMI containing 10% FBS.

3.2.1.4 Flow cytometry

Flow cytometry is a technique that allows the analysis of different cells in a cell mixed population, as well as to phenotypically characterize these cells based on their size, granularity and cell surface or intracellular expression of different molecules.

This technique is commonly performed by staining the cells with fluorochrome-labeled antibodies that are specific for molecules expressed in their surface or being produced by them. The flow cytometer is an instrument capable of detecting fluorescence on individual cells in a suspension and thereby determining the number of cells expressing the molecule labeled with the specific fluorochrome-labeled antibody. A representative scheme of the principle of flow cytometry is depicted in Fig. 16 (from Abbas and Lichtmann in: Cellular and Molecular Immunology).

Spleen and decidual cells were analyzed in order to determine the systemic (in spleen cells) and local (in decidua cells) immune response after the different treatments.

Extra- and intracellular staining for tissue samples

Isolated spleen or decidual lymphocytes were incubated for 1 hour with 50 ng/ml PMA and 1 µg/ml ionomycin at 37°C with 5% CO_2 for stimulation of cytokine secretion. The adition of PMA and the ionophore ionomycin is a powerful activating stimulus acting on protein kinase C and calcium ion influx, and is used both to induce cytokine expression of cells previously activated by physiological stimuli (Pala *et al.*, 2000). After this, 2 µM of monensin, which acts by blocking the transport between the endoplasmic reticulum and the Golgi complex (Luttmann *et al.* in: The Experimentator Immunologie), was aggregated and incubated for further 3 hours to allow intracellular accumulation of secreted proteins. Cells were further washed and antibodies against surface markers were incubated for 10 min at 4°C in darkness.

For fixation, paraformaldehyde solution (PFA) at a concentration of 1% (p/v) was used, and cells were incubated overnight (O.N.) at 4°C. After washing the cells, antibodies for the detection of intracellular proteins were incubated 20 min at 4°C in darkness, diluted in saponin 0.1% (p/v), a detergent solution for permeabilization of the cells. After this incubation, cells were washed with saponin solution, in order to remove excess of intracellular antibodies. The labelled cells were finally resuspended in FACS buffer and analyzed in a FACS Calibur (Becton Dickinson) cytometer. The lymphocyte population was gated based on size and granularity and used for further analysis. When only analysing extracellular markers, incubation with PMA, ionomycin and monensin was avoided.

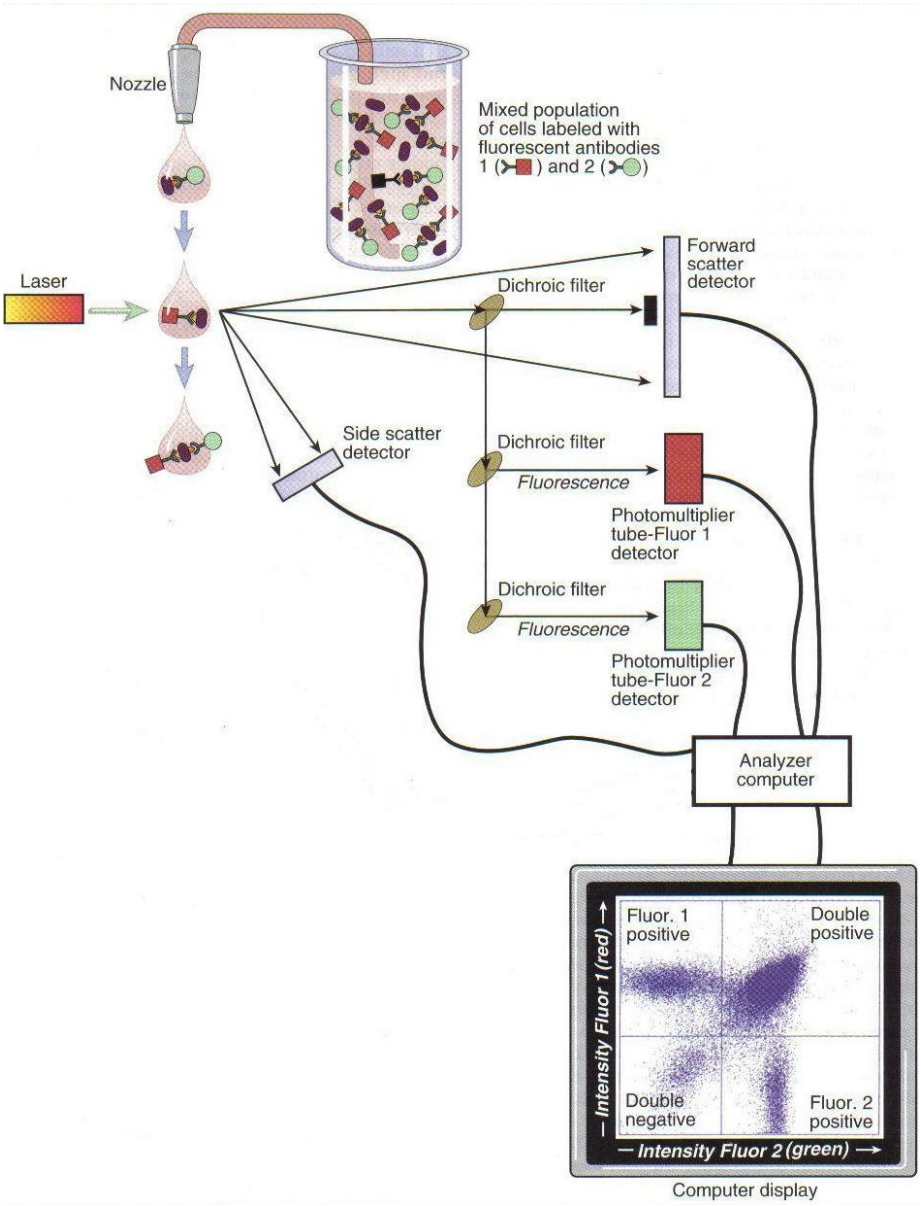


Fig. 16 : Principle of flow cytometry.

Figure modified from Abbas and Lichtmann, 2003. Briefly, cells pass one at a time through a fluorimeter with a laser-generated incident beam and the light that emerges from the sample is analyzed for forward and side scatter (size and granularity, respectively) as well as fluorescent light of two or more wavelengths that depend on the fluorochrome labels attached to the antibodies. The example represented here is based on a staining of two antigenic markers.

All washing steps were performed using FACS washing buffer (1% bovine albumin serum in PBS). Samples were analyzed in a FACS Calibur (Becton Dickinson, Heidelberg, Germany). Negative controls were performed by using the respective isotype controls, and cells without antibodies were also included as negative controls.

The extra- and intra-cellular markers analyzed included:

Extra-cellular markers:

CD3: PerCP anti-mouse CD3e (CD3ε chain), diluted 1:100

CD4: detected with FITC anti-mouse CD4 (L3T4), diluted 1:100

CD8: detected with PE-Cy5 anti-mouse Cd8a (Ly-2), diluted 1:100

CD69: detected with PE-Cy7 labeled anti-mouse CD69 (VEA Antigen) (H1.2F3)

CD95: detected with PE-Cy7 labeled anti-mouse CD95 (Jo2)

Intra-cellular markers:

IL-10: detected with PE-rat anti-mouse IL-10, diluted 1:200

IL-4: detected with PE-rat anti-mouse IL-4, diluted 1:200

TNF-α: detected with PE labelled anti-mouse TNF-α, diluted 1:200

IFN-γ: detected with PE anti-mouse IFN-γ, diluted 1:200

3.2.1.5 DNA Isolation

Placenta, liver and fetuses from animals receiving adenoviruses were snap-frozen in liquid nitrogen and kept at –80°C until use. DNA extraction from tissue homogenates was performed using the peqGOLD Tissue DNA Mini Kit. For that, livers, fetuses and placentas (30 mg of tissue or less) were grinded in a mortar using a pistil under the influence of liquid nitrogen. Samples were taken up in TL Buffer, and after adding OB™ protease, samples were incubated at 55°C in a shaking water bath for at least three hours. After that, isolation was performed following manufacturer´s instructions, using HiBind® DNA columns for the specific binding of DNA. After two washing steps, samples were eluted with elution buffer and kept at -20°C until further use.

3.2.1.6 Measurement of adenoviral particles in tissues by Real Time PCR

SYBR Green chemistry is an alternate method to the method using fluorescent probes used to perform real-time PCR analysis. SYBR Green is a dye that binds the Minor Groove of double stranded DNA (Applied Biosystems Manual). When SYBR Green dye binds to double stranded DNA, the intensity of the fluorescent emission increases. As more double stranded amplicons are produced, SYBR Green dye signal will increase. The SYBR Green dye will bind to any double stranded DNA molecule, while the 5′ Nuclease assay (explained later) is specific to a pre-determined target. For this reason, primers designed for this methodology have to be very specific and should not form any dimmers. To analyze whether this occurs, it is necessary to perform a melting curve, which plots the fluorescence as a function of the

temperature as the thermal cycler heats through the dissociation temperature of the product. The shape and position of this melting curve are functions of the GC/AT ratio, length and sequence and can be used to differentiate desired and undesired amplification products (Ririe *et al.*, 1997).

In the real time PCR methodology, the reactions are characterized by the time point during cycling when amplification of a PCR product is first detected. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in the fluorescence is observed. The parameter C_T (threshold cycle) is defined as the fractional cycle number at which the reporter fluorescence generated by the cleavage of the probe passes a fixed threshold above base line.

Amplifications reactions (13 µl) were performed by mixing 1 µl DNA, 6,25µl Master mix (Stratagene, Heidelberg, Germany) containing PCR Buffer, dNTPs, $MgCl_2$, Ampli-Taq DNA Polymerase and SYBR-Green, 3 µl of the primer mix and 2,75 µl water. PCR reaction was performed as follows: 2 min at 50° C followed by an initial denaturation step of 10 min at 95°C, followed by 15s at 95°C and 1 min at the appropriate annealing temperature (60°C) for 40 cycles. The measurement of adenoviral particles was performed by detecting GFP or viral constructs. All reactions were performed on the ABI Prism 7700 Sequence Detection System (Perkin Elmer Applied Biosystems). As intra-laboratory controls, non-related DNA samples were checked for their viral expression showing negative values. The sequences of the primers used are shown in Table 1.

Table 1 : Sequences of the primers used for GFP detection by real time PCR

	Primer sequence (5´→3´)
Apob	fw: CGTGGGCTCCAGCATTCTA
	rev: TCACCAGTCATTTCTGCCTTTG
GFP	fw: TGCAGTGCTTCAGCCGCTA
	rev: AAGATGGTGCGCTCCTGGA

The primers for GFP were used at a concentration of 900 nM for forward and 900 nM for reverse primers. The primers for Apob were used at a concentration of 300 nM for forward and 300 nM for reverse primers. Optimal primer concentrations were determined by titration of the primers with DNA samples from a GFP^+ animal (titration done by Nadja Ahmad during

her Diploma Thesis in our laboratory), where the expression of GFP was linked to Apo-b expression.

3.2.1.7 Pre-treatment of slides with 3-aminopropyltriethoxysilane (APES)

Solutions of APES are normally used to treat glass slides. The silane reacts with the –OH group of glass, covalently linking aminoalkyl groups to the glass. The resultant surface acts "sticky", promoting the binding of the tissue to the glass. For the treatment of the glass slides, they were soaked in a solution of 2% APES in methanol and washed twice with distilled water. Slides were dried completely in the oven and were then ready to use for paraffin as well for cryosections.

3.2.1.8 Detection of GFP by fluorescence microscopy

In order to detect the expression of the green fluorescent protein by fluorescence microscopy, placental tissues as well as placental tissue bound to fetuses were cut in cryostat in 8 μm thick samples. After fixing the samples for 10 min with PFA 4%, samples were dried and kept at -20°C until use. For analysis of GFP expression, samples were mounted with DAPI and observed under fluorescence microscope.

3.2.1.9 Paraffin embedding of the tissues

Paraffin embedding of the tissues was performed following a well-established protocol (Saint-Marie, 1963). For doing that, tissues were fixed in 96° ethanol at 4°C for 1-7 days, following the proportion of 50 ml alcohol/g tissue. After that, following incubations were performed:

Dehydratation:	-Ethanol 100° at 4°C, 1-2 h, with 4x changes
	-Xylol at 4°C, 1-2 h, with 2 changes
	-Xylol at RT, 1-2 h, with 1x change
Inclusion:	-Paraffin at 56°C, 1-2 h

After inclusion in paraffin, tissues were kept at 4°C.

For cutting the tissues, a microtome was used, and all tissues were cut in 5 μm thick specimens and placed in slides previously covered with 3-aminopropyltriethoxysilane. Samples were allowed to dry at 37°C O.N. and were finally kept at R.T.

3.2.1.10 Dewaxing of the tissues

In order to perform an immunostaining, it is necessary to first remove the paraffin in the cuts. For doing that, the following incubations were performed:

Dewaxation:	Xylol, 2 x 20 min
Fixation/Hydration:	Ethanol 100°, 10 min
	Ethanol 95°, 10 min
	Ethanol 75°, 10 min
	Distilled water, 5 min

3.2.1.11 Hematoxilin-Eosin staining

In order to analyze the morphology of the tissues (placenta, decidua and resorptions), a staining with Hematoxylin and Eosin, which stain nucleus and cytoplasm of the cells respectively, was performed. For doing this, following incubation steps were performed:

- Distilled water, 5 min
- Hematoxylin, 2 min
- Tap water, 5 min
- Eosin, 10 sec.
- Ethanol 75°, 10 sec, with agitation
- Ethanol 95°, 10 sec, with agitation
- Ethanol 100°, 2 x 10 sec, with agitation
- Xylol, 2 x 5 min

After that, slides were mounted with Roti-Histokitt and analyzed under light microscope.

3.2.1.12 Immunohistochemistry (IHC)

Immunohistochemistry (IHC) consists in the detection of specific antigens in histologic tissue sections by the use of a system consisting in a specific antibody for the antigen of interest. This enzyme coupled system allows the conversion of a colorless substrate into a colored insoluble substance that precipitates at the site where the antibody an thus the antigen are localized (Abbas and Lichtmann in: Cellular and Molecular Immunology). The specific antibody

can be directly coupled to the enzyme, or a secondary antibody recognizing the first specific antibody can be the one coupled to the enzyme. Another possibility, normally used to amplify the signal and thus detect low expressed antigens, is the use of a specific primary antibody, which is recognized by a secondary antibody coupled to biotin. This biotin is then recognized by avidin coupled to an enzyme, normally horse radish peroxidase (HRP). This methodology is normally known as ABC system (Avidin-Biotin-Complex), and was routinely used in the experiments done in the course of this doctoral thesis.

IHC was performed for each molecule of interest on tissue pieces containing placenta and decidua. After dewaxation, the sections were washed with Tris buffered saline solution (TBS, pH = 7.40) for 10 min and treated with 3% hydrogen peroxide in methanol for 30 min at RT to block the endogenous peroxidase activity, which is very high in placental tissue. The tissues were then washed and exposed to 5% BSA in TBS for 20 min at RT for protein blocking, stained with the primary antibody (Ab) diluted in 5% BSA in TBS, and incubated O.N. at 4°C. The tissues were then washed and further stained with the secondary Ab diluted in 5% BSA for 1 h at RT. After washing, the samples were incubated for 30 min with an AB-Complex/HRP solution. Finally, the sections were developed with AEC+ Substrate Chromogen, counterstained with Hematoxylin and mounted. Negative controls were obtained by replacing the first antibody with 5% BSA or 10% rabbit serum.

The dilutions of the primary and secondary antibody used in each case are indicated in Table 2.

Table 2 : Dilutions of the antibodies used for immunohistochemical staining

Molecule of interest	***First antibody***	***Secondary antibody***
HO-1	1:100	1:100
HO-2	1:500	1:100
CD3	1:20	1:100
VEGF	1:100	1:200

For the analysis of the results, samples were analyzed under a light microscope, by counting the number of positive cells (for VEGF and CD3 staining). In these cases, where only some cells were positive for the markers, the number was determined counting the number of cells per field. Each field consisted in a 0.25 mm^2 square coupled to the ocular of the microscope. Using a magnification of 200X (20X objective and 10X ocular magnification), at least 20 representative fields per sample were counted, and the results were expressed for each sample as the mean of cells per mm^2. The analysis was performed without knowing the nature of the samples and was confirmed by an independent observer.

In the case of HO-1 and HO-2 staining, where almost all cells were stained for HO-1 and HO-2 expression, the analysis was performed for each cell type independently. The expression levels were set by using scores from 0 (no staining) to 6 (very intense staining) for every cell type without knowing the nature of the samples, and the results were again confirmed by an independent observer.

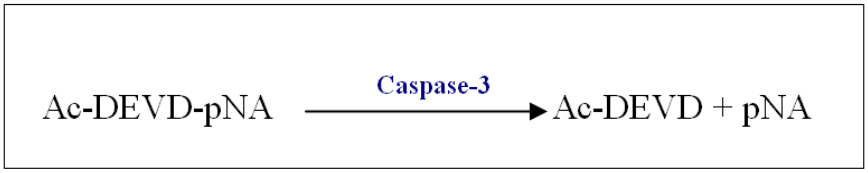
3.2.1.13 Protein isolation

For protein extraction from tissues, frozen specimens were re-suspended in 500 µl Lysis-buffer containing CHAPS, HEPES and DTT. The buffer used was the one recommended for caspase activity measurement (explained later). The samples were homogenized using a glass homogenizer. After isolation, homogenates were centrifuged for 10 min at 10.000 g, and the supernatant containing the proteins was transferred to a fresh tube. Protein concentration was assessed using the BioRad Protein Assay as indicated by the manufacturer. Protein samples were kept at -80°C and when working with them, they were always kept on ice.

For isolation of proteins from cells, 5 x 10^5 cells were washed with PBS and resuspended in 50 µl of Lysis Buffer containing Protease Inhibitor and used immediately or kept frozen at -70°C until use. For the preparation of the samples, the cell suspension was centrifuged 5 min at 15000 g at 4°C and the supernatant containing the proteins was further transferred to a fresh tube.

3.2.1.14 Measurement of Caspase-3 Activity

The caspase-3 colorimetric assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase-3, resulting in the release of the p-nitroaniline (pNA) moiety by the following reaction:



For doing this assay, placentas were snap-frozen in liquid nitrogen and kept in –80°C until use. For extracting proteins, a lysis buffer containing 5 mM CHAPS, 50 mM HEPES and 5 mM DTT was used. It is very important that the proteins are lysed in a buffer without protease inhibitors, since they can interfere in the reaction, especially cysteine protease inhibitors. The determination of total protein concentration was performed using the Bio-Rad Protein Assay Dye Reagent with a calibration curve performed with bovine serum albumin standards. The determination of the caspase-3 activity was performed in 96-well plates by means of the colorimetric Caspase-3 Assay Kit (Sigma) using 50 µg of total protein/sample and following manufacturer's instructions. Three controls were included: an inhibitor-treated lysate control for measuring the non-specific hydrolysis of the substrate, a caspase-3 positive control provided with the kit, and a reagent blank. Briefly, the following schema was followed (Table 3):

Table 3 : Scheme of the procedure for the determination of caspase-3 activity.

	Cell lysate	Caspase 3 2 µg/ml	1x Assay Buffer	Caspase-3 Inhibitor	Caspase-3 Substrate
Reagent Blank	-	-	90 µl	-	10 µl
Sample	5 µl	-	85 µl	-	10 µl
Sample + Inhibitor	5 µl	-	75 µl	10 µl	10 µl
Caspase-3 positive control	-	5 µl	85 µl	-	10 µl
Caspase-3 positive control + Inhibitor	-	5 µl	75 µl	10 µl	10 µl

For each sample, a control using an inhibitor was used. Positive controls as well as reagent blanks were included in each plate assayed.

The test was performed in 96-well plates. Each setting was made in duplicate for each sample. Plates were incubated O.N. at 37°C, and the optical density (O.D.) was finally measured at 405 nm.

The calibration curve was made using a p-nitroaniline standard provided by the manufacturer. The concentration of the stock solution was determined by measuring the O.D. at 405 nm and

calculating it taking into account the ε^{mM} of 10.5. In each plate, the calibration curve was done in duplicate in serial 1:2 dilutions ranging from 200 µM until 12.5 µm.

For calculating the Caspase-3 Activity, the following formula was used:

$$\text{Activity, µmol pNA/min/ml} = \frac{\mu mol\ pNA \times d}{t\,x\,V}$$

where:

t= incubation time (min); d= dilution; V= volume of sample in ml

µmol pNA was calculated for each well using the pNA calibration curve.

3.2.1.15 In situ cell death detection by TUNEL technology

The cleavage of genomic DNA during apoptosis may yield double-stranded, low molecular weight DNA fragments as well as single strand breaks, or nicks, in high molecular weight DNA. Those DNA strand breaks can be identified by labelling free 3'-OH termini with modified nucleotides in an enzymatic reaction. The *In Situ* Cell Death Detection Kit labels DNA strand breaks by Terminal deoxynucleotidyl transferase (TdT), which catalyses the polimerization of labeled nucleotides to free 3'-OH DNA ends in a template-independent manner (TUNEL reaction). The incorporated fluorescein is detected by anti-fluorescein antibody Fab fragments, conjugated with horse-radish peroxidase (POD). After substrate reaction, stained cells can be analyzed under light microscope.

The detection of apoptotic cell death at single cell level was performed in tissues containing placenta and decidua following manufacturer's instructions. However, a modification of the temperature of incubation was performed. In the current protocol, the TUNEL reaction mixture and the Converter-POD were incubated at RT instead of 37°C. The modification was performed based on the observation of unspecific staining of the cytoplasm of the cells when using the 37°C incubation while establishing the staining. Briefly, paraffin sections were dewaxed and hydrated as usual, followed by a permeabilization of the samples. For that, slides were treated with citrate buffer pH 6 for 5 min at 600 W in a microwave. After cooling of the slides, they were washed twice with PBS. The endogenous peroxidase activity of the samples was blocked with 3% H_2O_2 in methanol for 30 min at RT. After washing the slides twice with PBS, a protein blocking was performed during 20 min at RT using a buffer containing

3% BSA and 20% SFB in Tris-HCl pH 7.5. The TUNEL reaction mixture was added to the samples, and incubated in a humified chamber for 60 min at RT. For negative controls, label solution instead of TUNEL reaction mixture was added. After the incubation, the samples were washed twice with PBS, and a Converter-POD solution was added. After 30 min of incubation at RT, tissues were washed twice with PBS and developed using the AEC+ Substrate Chromogen, counterstained with Hematoxylin and mounted with Aquatex.

For the quantification of the number of apoptotic cells, stained nuclei were identified and counted per field. Each field consisted in a 0.25 mm^2 square coupled to the ocular of the microscope. Using a magnification of 200X (20X objective and 10X ocular magnification), at least 20 representative fields per sample were counted, and the results were expressed for each sample as the mean of cells per mm^2. The analysis was performed without knowing the nature of the samples and was confirmed by an independent observer.

3.2.1.16 SDS-Polyacrilamide gel electrophoresis (SDS-PAGE)

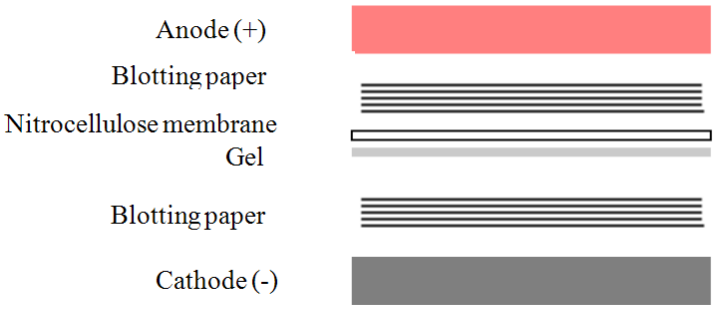
When an electric field is applied to a solution containing protein molecules, these molecules migrate in a direction and at a speed that reflects their size and net charge. This is the basis of the technique called *electrophoresis* (Alberts *et al*. in: Essential Cell Biology, 2004). When proteins are mixed with negatively charged molecules of sodium dodecyl sulfate (SDS), the negatively charged SDS-protein complex will migrate through a porous polyacrilamide gel to the anode. When protein samples are treated like this, they will migrate at a rate that reflects only their molecular weight, since they are all be negatively charged by an excess of SDS. A reducing agent as like mercaptoethanol is usually added to break any S-S linkage in or between proteins.

Proteins isolated from tissues or from cells were mixed with a sample buffer containing SDS, β-mercaptoethanol and bromophenol blue and heated for 5 min at 95°C in order to allow the complete denaturalisation of the proteins. The same procedure was carried out with the protein marker. Samples were transferred to an acrylamid/polyacrylamid running gel with a 5% stacking gel. Electrophoresis was performed at 150 V until its end.

3.2.1.17 Western Blot

Blotting is the technique of transferring electrophoretic products onto other materials prior to visualisation. Western Blot, specifically, is the transfer (or blotting) of proteins to a membrane (Acquaah in: Understanding Biotechnology, 2004). The membrane is then sequentially ex-

posed to solutions containing a primary antibody, followed by a secondary antibody to which an enzyme or biotin is coupled. Here, after finishing of the SDS-PAGE, proteins were transferred into a nitrocellulose membrane. For doing this, the following schema was followed:



When using the semi-dry blot method, the protein transfer took place during 30 min at 250 mA. When using the wet blot method, protein transfer took place during 2 h at 180 mA. For verifying the full protein transfer, the nitrocellulose membranes were stained with Ponceau Red for 3 min, and further decoloured using distilled water (dH_2O).

The expression of HO-1 was analyzed (in the membrane) by immunoblot as follows:

The membrane was blocked with 5% milk powder in TBS for 1h at R.T., and further washed with TBST, 3 x 5 min each time. The first antibody (rabbit anti rat-HO-1 polyclonal antibody, diluted 1:2000 in 5% milk in TBS) was applied for 2 h at R.T. After washing with TBST, the samples were incubated with the secondary antibody (anti-rabbit horseradish peroxidase, diluted 1:5000 in 5% milk in TBS) for 1 h at R.T. The membrane was washed again, and the reaction was finally developed by using chemoluminiscence (ECL Assay of Amersham) and exposed onto Kodak Miomax MR Imaging film. The intensity of the bands was quantified by using the Quantity One® Software, Version 4.5.2 from Bio-rad.

3.2.1.18 RNA Isolation

One placenta and decidua of each animal were snap-frozen in liquid nitrogen and kept in -80°C until preparation. RNA extraction was performed using Trizol Reagent, which is a monophasic solution of phenol and guanidine isothiocyanate, and represents an improvement to the single step RNA isolation method developed by Chomczynski and Sacchi (1987). Briefly, tissues were homogenized in Trizol using an Ultra-Turrax T25 homogenizer. After adding chloroform and vortexing for 2 min at RT, samples were centrifuged at 10.000 g for 10 min at 4°C. The upper phase obtained after the centrifugation was then transferred to a new tube, and

ice-cold ethanol was added. After an incubation of 10 min at -20°C, samples were centrifuged for 10 min at 10.000 g at 4°C. The pellet obtained after this centrifugation was then washed three times with ethanol 80°, in order to remove the rest of Trizol reagent. Between each wash step, cells were centrifuged for 10 min at 10.000 g and 4°C. After the last wash, the pellet was allowed to dry and it then was resuspended with RNase free water. In case that the pellet was not immediately resuspended, samples were kept at 4°C to allow pellet resuspension. RNA concentration was determined by measuring the O.D. at 260 nm and at 280 nm. To calculate the RNA concentration, the following formula was used:

$$\text{RNA concentration} = A_{260\,nm} * 40\ mg/ml * \text{Dilution}^{-1}$$

3.2.1.19 cDNA Synthesis

Samples containing 2 µg of total RNA were placed for 2 min on ice and added with dNTPs (2,5 mM), Amersham Pharmacia), DNase I (2U/µl, Stratagene) and RNase-Inhibitor (40 U/µl) mixed in reaction buffer. The mix was incubated for 30 min at 37°C and further heated to 75°C for 5 min. The addition of the reverse transcriptase (200 U/µl) and RNase-inhibitor in distilled water started the reverse transcription. This reaction mixture was incubated at 42°C for 60 min followed by incubation at 94° C for 5 min.

As negative controls, we performed a so called RT^- control, which consists in the same mixture, but avoiding the aggregate of Reverse Transcriptase. This control was done in order to check the samples for contamination with genomic DNA.

Once the cDNA synthesis was completed, the samples were immediately used or kept at -20°C.

3.2.1.20 TaqMan™PCR

The Real-Time TaqMan™ PCR is a sensitive, reproducible and specific method for the quantification of mRNA. Together with the specific primers for the gene of interest, an oligonucleotid fluorescence probe is used. The probe is marked at the 5´end with a fluorescent reporter-dye (6-Carboxyfluorescein, FAM) and at the 3´end with a quencher-dye (6-carboxy-tetramethyl-rodhamin, TAMRA). While the probe is intact, the proximity of the quencher reduces the fluorescence emitted by the fluorescent dye. During the PCR, if the target sequence is present, the probe anneals downstream from one of the primer sites and is cleaved by the

5´-exonuclease activity of Taq polymerase as the primer is extended. Then, the cleavage of the probe separates the reporter dye from the quencher, increasing the reporter dye signal. Additional reporter dye molecules are cleaved with each cycle, which produces an exponential increase in the fluorescence intensity that is proportional to the amount of the amplicon produced (Fig. 17).

In the real time PCR methodology, the reactions are characterized by the time point during cycling when amplification of a PCR product is first detected. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in the fluorescence is observed. The parameter C_T (threshold cycle) is defined as the fractional cycle number at which the reporter fluorescence generated by the cleavage of the probe passes a fixed threshold above base line.

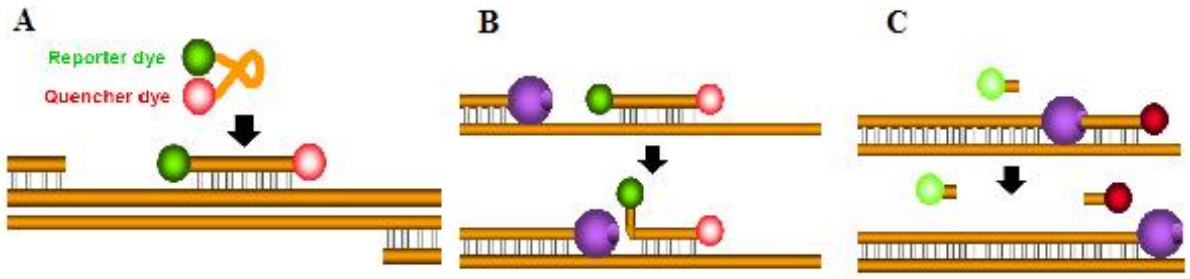


Fig. 17 : Principle of the TaqMan™ PCR Reaction (Figure taken from the Applied Biosystems´s user manual)

A) The 5´nuclease assay

B) Polymerase collides with TaqMan® probe

C) Cleavage of the TaqMan® probe

The PCR reaction was developed using the ABI PRISM™ 7700 Sequence Detection Systems, which detects the fluorescence intensity.

The primers and probes were designed using the Primer Express Software. The establishment of the primers, their titration and their efficiency determination through standard curves were performed at the Institute of Medical Immunology (Charité, Berlin) by Katrin Vogt, a member of the Institute. The sequences for primers and probes are detailed in Table 4.

For each sample, 1 µl of template was used together with 6 µl primer mix and 1 µl probe. The final volume of 25 µl was reached aggregating 12.5 µl Mastermix and 4.5 µl distilled water.

For the PCR, the following cycle conditions were applied:

50°C 2:00 min
95°C 10:00 min
95°C 0:15 min
60°C 1:00 min

40 cycles for each determination were performed.

Table 4 : Sequences of primers and probes

	Primer (5´→3´)	***Probe (5´→3´)***
β-actin	Fw: GCTTCTTTGCAGCTCCTTCGTT	CAGCCTTCCTTCTTGGGTATGGAATCCT
	rev: GTTGTCGACGACCAGCGC	
Bag-1	fw:GCTAACCACCTGCAAGAATTGAAT	TTCTGACATCCAGCAGGGTTTTCTGGC
	rev: GTTTGCAGAGAGCCTCCGC	
Bcl-2	fw: TGAACCGGCATCTGCACA	AACGGAGGCTGGGATGCCTTTGTG
	rev: CAGAGGTCGCATGCTGGG	
Bcl-xl	Fw: GGTGAGTCGGATTGCAAGTTG	CCTGAATGACCACCTAGAGCCTTGGATCC
	r:GTAGAGATCCACAAAAGTGTCCCAG	
HO-1	fw: GGCTACCATGCCAACTTCTGTCT	CACACAGTACAGCAAGGTCCTTGCCCT
	Rev: CCGGGTTGTGTTGGTTGTAGA	
CD3	fw:ATTGCGGGACAGGATGGAG	TCGCCAGTCAAGAGCTTCAGACAACGA
	rev:CTTGGAGATGGCTGTACTGGTCA	
FoxP3	Fw: CCCAGGAAAGACAGCAACCTT	ATCCTACCCACTGCTGGCAAATGGAGTC
	Rev: TTCTCACAACCAGGCCACTTG	
TGF-β	fw: GGCTACCATGCCAACTTCTGTCT	CACACAGTACAGCAAGGTCCTTGCCCT
	Rev: CCGGGTTGTGTTGGTTGTAGA	

Sequences are indicated in a 5´→3´direction

3.2.1.21 Relative RNA Quantification

A relative quantification of the mRNA expression is possible because of the use of a “house keeping gene”, which is constitutively expressed in all cell types. In all cases, β-actin was used as house keeping gene after confirming its constitutive expression in the analyzed tissues (decidua and placenta).

The determination of the ΔC_T value is made using the formula:

$\Delta C_T = C_T$ (gene of interest) – C_T ("house keeping gene")

Due to the inverse proportional relationship between the Ct and the original gene expression level, and the doubling of the amount of the product with every cycle, the original expression level for each gene of interest is expressed as following:

$$mRNA = 2^{-\Delta CT}$$

3.2.2 Studies using HO-1 over-expressing primary T cells

3.2.2.1 Culture of primary T lymphocytes

Spleen and lymph nodes were isolated from CBA/J females. Cells from spleen and lymph nodes were separately isolated. For spleen cells isolation, spleen tissues were crushed against a 100 μm net, collected in medium containing 10% FCS and the erythrocytes were lysed with lysis buffer containing NH_4Cl, $KHCO_3$ and EDTA for 10 min at R.T. After centrifugation, cells were washed once with complete medium and finally resuspended in RPMI containing 10% FBS, Na-Piruvate, Penicillin, Streptomycin and β-mercaptoethanol and kept on ice until use. For lymph node cell isolation, lymph nodes were crushed against a 100 μm net, washed with complete medium, and after centrifugation resuspended in RPMI containing 10% FBS, Penicillin, Streptomycin, Na-Piruvate and β-mercaptoethanol.

Different culture conditions were tested: a) stimulation of the cells with irradiated APCs from the male (DBA/2J) or b) polyclonal stimulation with anti-CD3 and anti-CD28. For stimulation with male antigens, spleen and lymph node cells from DBA/2J were irradiated at 30 Gy, and immediately used. The cell amount was always the same as the one from the CBA/J female cells. For polyclonal stimulation, 1μg/ml anti-CD3 and 0.1 μg/ml anti-CD28 were used. In both cases, 10 ng/ml of recombinant murine IL-2 (rmIL-2) were added to the culture (final concentration). Cells were routinely maintained at 37°C in a humified incubator with 5% CO_2. When necessary, medium was changed for fresh medium containing rmIL-2 (normally at a 2-3 days interval). After one week in culture, cells were restimulated with either APC or anti-CD3/anti-CD28. The optimal cell amount was tested and 1.5 x 10^6 cells/ml were finally used.

The effect of antibodies against Th1 phenotype was also tested in our culture at a concentration of 5 μg/ml anti-IFN-γ and 5 μg/ml anti-TNF-α. In another setting, IL-4 was added to the cultures at a 10% of the final volume. IL-4 was obtained as a supernatant of the 3T3 BMG IL-4 cell line (kind gift of Prof. Werner Müller, Braunschweig). This was done in order to obtain majority of Th_2 cells.

3.2.2.2 Generation of retroviral vectors expressing HO-1

Two different retroviral vectors expressing HO-1 were generated. Both retroviral vectors encode for the rat HO-1 gene, while the second construct express in addition the EGFP gene for tracing the transduced cells. The generation of these constructs was done according to standard procedures. Briefly, the rat HO-1 cDNA was used as template for PCR-amplification with specific primers introducing Xho-1 restriction sites on both ends of the PCR product. The resulting PCR-fragment was subsequently cloned into the pdrive cloning vector (Qiagen) to allow DNA-sequencing. Then, the HO-1 fragment was excised from pdrive and cloned into the pLXSN retroviral vector (Clontech). The correct orientation of the HO-1 transgene was confirmed both by restriction analysis and DNA-sequencing. For the generation of the bicistronic retrovirus expressing both HO-1 and EGFP, a pLXSN-based construct containing an internal ribozyme binding site (IRES) followed by the EGFP-gene (kind gift of Dr. A. Flügel, Max-Planck-Institute of Neurobiology, Martinsried) was employed. A similar strategy as described for the generation of pLXSNHO-1 was performed to obtain the bi-cistronic construct. The generation of both vectors was performed previously to the beginning of this thesis by Katrin Vogt, member of the Institute of Medical Immunology.

3.2.2.3 Establishment of the packaging cell lines

The retroviral gene transfer system needs basically two components: the packaging cell line and the retroviral vector. The retroviral vector is modified, being the genes *gag*, *pol* and *env* deleted. It contains the packaging signal Ψ^+, and the 5 - and 3 –LTRs (Long Terminal Repeats), which flank the transgen and a selection marker. The packaging cell line contains the genes *gag*, *env* and *pol*. Once the packaging cell line is transfected with the retroviral vector, it is able to produce retroviral particles which are able to infect other cells, but are unable to replicate by themselves.

The packaging cell line used was one derived from the NIH/3T3, called GP+E 86 (Dr. Flügel, Neurobiology, Munich). It was cultured under normal conditions, in T75 cm^2 flasks, using

DMEM with 10% FCS, penicillin, streptomycin and glutamine, in an incubator with 5% CO_2 at 37°C. Routine passaging of the cells was performed using brief exposure to a trypsin-EDTA solution followed by vigorous agitation of the culture flask. The cells were frozen employing a solution containing 90% FCS and 10% DMSO.

3.2.2.4 Transfection using calcium phosphate

24 h before transfection, $2.2x10^6$ GP+E 86 cells were seeded in 60 mm (21,5 cm^2) dishes in DMEM with 10% FCS, in order to allow about 60-70% confluence on the next day. From two to four hours before the transfection, culture medium was replaced by 5 ml of fresh medium. The preparation of the phosphate-DNA suspension was made using 15 µg Plasmid DNA, 31 µl 2M $CaCl_2$, ad 250 µl distillate water and 250 µl 2x HBSP-Buffer. After 15 min at R.T., the suspension was mixed and dispensed drop by drop onto the cells. 12-24 h after the transfection, medium was replaced for one containing 1 mg/ml G-418 in order to select the G-418 resistant cells.

Two different constructs, the pLXSN-HO-1, and the pLXSN-HO-1iresEGFP, were used. The efficient transfection and the over-expression of HO-1 in the both cell lines were verified by Western Blot analysis. Cells transfected with the HO-1iresEGFP vector were routinely checked for their EGFP expression under fluorescence microscope.

3.2.2.5 Limiting dilution

After verifying the HO-1 transgene expression through Western Blot, a limiting dilution method was used to obtain monoclonal cell lines from the polyclonal packaging cell line. This approach offers the possibility of select those clones with an optimal viral titer and transgene expression. To perform this, the following schemas followed:

1 x 96 plates with 5 cells/well
2 x 96 plates with 1 cells/well
1 x 96 plates with 0.3 cells/well

After 4 weeks under selection, the positive clones were tested again through Western Blot for the transgene expression, and those ones with the higher HO-1 expression were selected and further analyzed for their viral titer.

3.2.2.6 Viral titer determination

The viral titer determination was analyzed following the protocol proposed by the manufacturer (Clontech). In brief, 5 x 10^5 GP+E 86 cells were harvested two days before of the beginning of the viral titer determination in a 25 cm^2 flask. One day before transfection, NIH 3T3 cells were plated at a density of 1.10^5 cells/well with 2 ml medium/well in 6-well plates.

Lastly, for viral titer determination, the supernatants of the packaging cell lines were filtered through 0.45 μm cellulose acetate filters. Serial dilutions of the supernatant were made, from 1.10^{-1} until 1.10^{-8}, and the NIH 3T3 cells were infected by adding 1 ml of the diluted virus medium (day 0). The dilution medium contained polybrene (final concentration = 8 μg/ml). The selection with G-418 began 24 h after the infection, followed by a medium change, on day 6. On day 12 after infection, the colonies were counted under the microscope, and the viral titer corresponded to the number of colonies present at the highest dilution which contains colonies, multiplied by the dilution factor.

3.2.2.7 Concentration of viral supernatants by means of centrifugation

Since the titer of retroviral particles was not always sufficient to achieve good percentages of transduction, a concentration of the viral particles was aimed by means of centrifugation of the supernatant containing the viral particles. For that, supernatants from the packaging cell lines were collected and filtered through 0.45 μm filters to remove possible dead cells o cell debris present in the supernatant. Filtered supernatants (15 ml) containing the retroviruses were transferred into Vivaspin 20 tubes with a 100000 MWCO PES membrane and centrifuged for 30 min at 3300 r.p.m. at 4°C. After this first centrifugation, 15 ml of filtered supernatant were further added and centrifuged for 45 min at 3300 r.p.m. Concentrated supernatants were immediately used.

3.2.2.8 Isolation and culture of cells from CBA/J females

T cells were isolated from spleen and lymph nodes from CBA/J females, and treated for 10 min at RT with lysis buffer containing ammonium chloride, potassium hydrogen carbonate and EDTA, and further washed twice with medium containing 10% FBS. These cells were then cultured under the presence of antigen presenting cells (APCs) from a DBA/2J male isolated similarly from spleen and lymph nodes and treated with lysis buffer. These APCs were irradiated at 30 Gray (Gy) and co-cultured with the CBA/J cells, putting the same cell number of CBA/J and irradiated DBA/2J cells.

After observing that these cells were poorly stimulated with DBA/2J cells, other stimulation condition was tested, namely stimulation with anti-CD3 and anti-CD28 antibodies. For that, cells were cultured in the presence of 1 μg/ml anti-CD3 and 10 ng/ml anti-CD28 and in the presence of 10 ng/ml rmIL-2. For maintenance of the culture, medium was changed every two days, and rmIL-2 concentration was maintained at the same concentration, but no further anti-CD3 or anti-CD28 was added to the culture. Although a cell suspension containing different type of cells was isolated, the stimulation protocols tested were aimed to stimulate only T cells to proliferate.

3.2.2.8.1 $CD4^+$ T cell isolation

$CD4^+$ T cells were isolated from lymph nodes of CBA/J females using the MACS $CD4^+$ T cell negative selection kit following the manufacturer´s instructions. Briefly, a cell suspension from lymph nodes was stained with a cocktail of streptavidin conjugated antibodies against all cells except $CD4^+$ T cells. Subsequently, cells were incubated with biotin conjugated ferro-magnetic microbeads. The suspension was passed through a magnetic column, allowing the $CD4^+$ T cells to be collected as a negative fraction. After centrifugation of the cells, the cell pellet was resuspended in T-cell media and cells were counted. A representative fraction of the sample was taken in order to analyze the purity of the isolated cells by flow cytometry.

$CD4^+$ T cells were then cultured at a concentration of 1.5 x 10^6 cells/ml in 12 well plates with 1.5 ml/well. Polyclonal stimulation was performed as previously described and cells were routinely maintained in medium containing 10 ng/ml rmIL-2.

3.2.2.8.2 $CD4^+CD25^+$ regulatory T cells isolation (Treg)

$CD4^+CD25^+$ T cells (known as Treg) and $CD4^+CD25^-$ T cells (reportedly T-effector cells) were isolated from thymus using the MACS $CD4^+CD25^+$ regulatory T cell isolation kit following manufacturer´s instructions. Briefly, cells were resuspended in MACS Buffer, followed by addition of Biotin-Antibody Cocktail. After 10 min of incubation at 4°C, MACS buffer, biotin microbeads and $CD25^-$PE antibody were added. Cells were additionally incubated for 15 min at 4°C and the reaction was stopped by adding MACS buffer. Cells were centrifuged at 300 g for 10 min, and the cell pellet was resuspended in MACS Buffer. Non-$CD4^+$ T cells were depleted by using a LD Column, which allows the $CD4^+$ T cells to pass through as a negative fraction. After centrifugation of the isolated $CD4^+$ T cell fraction, the magnetic labelling of $CD25^+$ cells was performed. For doing this, cells were resuspended in MACS buffer, and anti-PE microbeads were added. Cells were incubated in darkness for

15 min at 4°C, and the reaction was stopped by adding MACS buffer and centrifuging at 300 g for 10 min. Cells were resuspended in MACS buffer and the magnetic separation was performed in a MS column. $CD4^+CD25^-$ cells were allowed to pass through the magnetic column and collected in a tube, and after removing the column from the magnetic field, $CD4^+CD25^+$ cells were flushed out by adding MACS buffer. Both fractions were centrifuged at 300 g for 10 min, resuspended in T cell media and counted. Representative samples of each fraction were taken in order to analyze the purity of the isolated cells by flow cytometry.

3.2.2.9 Retroviral transduction of mouse T lymphocytes

The aim of this part of the study was to obtain T lymphocytes over-expressing HO-1. Since retroviral vectors are able to permanently transduce cells, different conditions were tested in order to obtain enough number of T cells over-expressing HO-1.

The following conditions were tested: co-culture of packaging cell line and T-lymphocytes, culture of T-lymphocytes with supernatant containing the retroviruses, and culture of the cells with a concentrated supernatant. Every condition is described below:

3.2.2.9.1 Co-culture of packaging cell line and T-lymphocytes

This co-culture was performed in order to have the packaging cell line constantly producing retroviral particles during the proliferation process of the T cells under stimulation with antigen presenting cells (APCs) or under the stimulation with anti-CD3 and anti-CD28 antibodies in the presence of rmIL-2. For that, cells from the packaging cell line were cultured O.N. in 96-well plated with U-bottom in a concentration from $1x10^3$ to $1x10^5$ (different conditions tested). Once adhered to the plate, the supernatant was taken and CBA/J cells (cultured under the conditions explained in 3.2.2.8) were added, at a concentration from 1.10^3 to $1x10^5$ (different conditions tested). After 48 h of co-culture in the presence of polybren (tested at 4, 6 and 8 μg/ml), supernatant containing cells in suspension was taken, aiming to let the packaging cells adhered to the plate. Cells previously obtained from CBA/J females were further cultured and analyzed for transduction efficiency.

3.2.2.9.2 Culture of T-lymphocytes with supernatant containing the retroviruses

In order to avoid contamination of the T-lymphocyte culture with cells from the packaging cell line, transduction was attempted by means of the supernatant containing the retroviral particles, following a protocol described by Hori *et al.* (Hori *et al.*, 2003). For that, 2.5 x 10^5 T

cells from a CBA/J female were cultured in the presence of 5 x 10^6 APCs from the male (DBA/2J), previously irradiated at 30 Gy in a 24 well plate, and after 24 h of culture half of the volume was replaced by virus supernatant, previously filtered through 0.45 μm filters and containing polybren (at a final concentration of 4, 6 or 8 μg/ml). This procedure was also tested stimulating cells with anti-CD3 and anti-CD28 as previously described (3.2.2.8), as well as in 12 and 96 well plates.

3.2.2.9.3 Culture of T-lymphocytes with concentrated supernatant containing the retroviruses

Since the transduction of total cells from CBA/J using the previous conditions was not successful, a new protocol was tested using $CD4^+$ T cells previously isolated using magnetic cell separation (as explained in 3.2.2.8.1). In brief, cells from CBA/J females were cultured for 24 h using anti-CD3/anti-CD28 stimulation in the presence of rmIL-2 in 12-well plates with a total volume of 1.5 ml/well with 1.5 x 10^6 cells/ml. 1 ml of cell supernatant was carefully removed, and 500 μl of concentrated retroviral supernatant as well as 500 μl of media containing rmIL-2, anti-CD3 and polybren were added. The final concentration of polybren in the culture was 4 μg/ml. The transduction took place for 24 h, and 1 ml of media of the cells was replaced for fresh medium containing anti-CD3 and rmIL-2. Cells were further incubated at 37°C and were eventually measured for HO-1iresEGFP expression by flow cytometry.

3.2.2.10 Protein Transfection

The protein transfection was performed by using Chariot™, which forms a non-covalent compound with the protein of interest, in our case HO-1. This complex bypasses the transcription-translation process associated with gene expression. The complex formed stabilizes the protein, and helps to protect it from degradation during the transfection process. Upon internalisation, the complex dissociates and the macromolecule is free to proceed to its target organelle.

No protocol was available for the transfection of primary lymphocytes, therefore various conditions were tested in this work and the protocol was adapted for $CD4^+$ lymphocytes in culture. Briefly, for each well to be transfected, 3.5 μl of Chariot were dissolved in 100 μl of sterile water, and 2 μg of the protein of interest (HO-1, Stressgen) was dissolved in 100 μl of PBS. These two solutions were mixed up carefully and incubated at RT for 30 min. During this incubation time, a specific number of cells (2 x 10^6 per well) were washed twice with PBS and further centrifuged. After incubation, the pellet of washed cells was resuspended in

200 µl of the Chariot-protein complex, and placed in a 12-well plate. After adding 150 µl of RPMI without serum, cells were incubated for 30 min at 37°C in a CO_2 incubator, and 1 ml of RPMI with serum was added to stop the protein transfection and to avoid the damage of the cells. Cells were further incubated for 30 min-1h and used.

Protein transfection using β-galactosidase (provided by the manufacturer of the transfection kit) was used as a positive control to determine the percentage of transfection. Although it has to be considered that both proteins differ in structure and the transfection may not be the same, it was used as an internal control to verify whether the transfection has worked, since it was no colorimetric method available to analyze whether HO-1 has entered the cells. In addition, for HO-1, the transfection efficiency was checked by analysing the HO-1 protein content by Western Blot.

3.2.2.11 Measurement of β-galactosidase activity by hydrolysis of fluorescein di-β-D-galactosidase (FDG)

The principle of the measurement of the β-galactosidase activity is based in the stepwise hydrolysis of fluorescein-di-β-galactosidase (FDG) by β-galactosidase (Huang, 1991), which is measured by flow cytometry. The measurement of the activity of the β-galactosidase was performed following a protocol described by Nolan *et al.*, 1988. The reaction was performed at 37°C. Briefly, up to 1 x 10^6 cells were resuspended in 100 µl pre-warmed media and placed in pre-warmed tubes. After that, 100 µl of a pre-warmed 2mM solution of FDG was added, and the reaction took place for 1 min 30 sec at 37°C. The reaction was stopped by adding 1.8 ml of ice cold medium and vortexing. After incubation of the cells for 1 h on ice protected from light, cells were placed in 5 ml Falcon tubes and washed with FACS Buffer. After centrifugation of the cells, they were fixed with 1% PFA O.N. After washing the cells, they were measured on a flow cytometer, being FDG detectable on FL-1H.

3.2.2.12 Mixed leukocyte culture (MLC)

The mixed leukocyte culture (MLC) test (Bain *et al.*, 1964, Bach and Hirschhorn, 1964) is widely used in the transplantation field in order to study the histocompatibility and cellular mechanisms of graft rejection. As pregnancy involves antigens from two genetically different organisms, MLC reactions are a useful tool to study the histocompatibility and the cellular mechanisms involved when cells from both organisms are put in contact with each other.

For this study, cells obtained from pregnant DBA/2J-mated CBA/J females (day 5 of pregnancy) were put in contact with mitomycin-treated DBA/2J cells. In this assay, $CD4^+$ T cells over-expressing HO-1 were also added in order to analyze the effect of HO-1 over-expressing lymphocytes on this *in vitro* setting. $CD4^+$ T cells receiving the same transfection procedure but without HO-1 were used as control. The study involved the following steps:

3.2.2.12.1 Treatment of stimulator cells with mitomycin C

Treatment of mitomycin C is known to arrest the cell cycle in the stimulating cells, so that they remain viable but are unable to proliferate or divide (Malinowski *et al*., 1992). Mitomycin C crosslinks with DNA and selectively inhibits DNA synthesis; RNA and protein synthesis are less affected, with a slowing in progress of the cells through the S and into the G_2 phase (Anderson and Williams, 1977).

DBA/2J male cells were used as stimulator cells and were therefore treated with mitomycin C. Spleen and lymph node from DBA/2J males were crushed against at 100 µm net and collected in RPMI. After lysis of erythrocytes with a lysis buffer containing NH_4Cl, $KHCO_3$ and EDTA, the cells were finally re-suspended in RPMI containing 10% FBS. For treatment of the cells with mitomycin C, they were washed twice with RPMI without FBS, and finally resuspended in RPMI without FBS containing 50 µg/ml of mitomycin C, at a ratio of 1.10^7 cells/ml. Cells were incubated for 30 min at 37°C with 5 % CO_2. The reaction was stopped by washing the cells three times with medium containing 10% FBS.

3.2.2.12.2 Staining of responder cells with CFDA-SE

In order to measure the proliferation of responder cells by flow cytometry, cells were first stained with CFDA-SE, which is a dye known to intercalate in the membrane of the cells.

Spleen and lymph node cells were isolated as previously described from DBA/2J-mated CBA/J females (day 5 of pregnancy). $CD4^+CD25^-$ and $CD4^+CD25^+$ cells were isolated from thymus of these animals as previously described (3.2.2.8.2). All these cells were stained as followed: after washing the cells twice with PBS to remove rest of FBS from the medium, cells were resuspended in medium containing 1 µM of CFDA-SE in PBS, at a ratio of 1.10^7 cells/ml. Cells were incubated for 1 min 30 sec and the reaction was stopped by adding RPMI containing 10% FBS. After two washes with RPMI with FBS the, cells were re-suspended in the same medium. The number of viable cells was assessed by means of trypan blue as it is known that many cells die during the procedure.

3.2.2.12.3 Protein transfection of $CD4^+$ T cells from CBA/J females

The protein transfection was performed as mentioned above. $CD4^+$ T cells were transfected with HO-1 protein and these cells will be mentioned as HO-1 over-expressing $CD4^+$ T cells in the following sections. $CD4^+$ T cells that were treated with the protein transfection reagent but with PBS instead of HO-1 will be referred from now on as $CD4^+$ control T cells. For this study, purified $CD4^+$ T cells that were already at least one week in culture were used.

3.2.2.12.4 Mixed leukocyte reaction (MLC)

3.10^5 CFSE-responder CBA/J cells ($HO\text{-}1^+$ or control cells) were put in contact with 3.10^5 mitomycin-treated stimulator DBA/2J cells in RPMI containing 10% FBS, in a total volume of 100 µl. CD4+ T cells over-expressing HO-1 or CD4+ control T cells were added at a concentration of 1.10^5 cells per well. The schema indicated in Table 5 was followed.

Table 5 : Scheme of reaction in 96 well plates

	$CFSE^+$ CBA/J ♀ cells	***DBA/2J ♂cells***	***$HO\text{-}1^+$ $CD4^+$ Tcells***	***$CD4^+$ control Tcells***
Spleen cells	1.10^5	1.10^5	-	-
	1.10^5	1.10^5	1.10^5	-
	1.10^5	1.10^5	-	1.10^5
Lymph node cells	1.10^5	1.10^5	-	-
	1.10^5	1.10^5	1.10^5	-
	1.10^5	1.10^5	-	1.10^5
$CD4^+CD25^+$ cells	1.10^5	1.10^5	-	-
	1.10^5	1.10^5	1.10^5	-
	1.10^5	1.10^5	-	1.10^5
$CD4^+CD25^-$ cells	1.10^5	1.10^5	-	-
	1.10^5	1.10^5	1.10^5	-
	1.10^5	1.10^5	-	1.10^5

Cells were cultured in a final volume of 100 µl/well in RPMI containing 10% FBS.

The cells were cultured for 48 h in 96-well plates, at 37°C in humified incubator with 5% CO_2 atmosphere. Each experiment setting was performed in triplicate, and the proliferation of $CFSE^+$ cells was analyzed by flow cytometry at two time points, namely 0 h and 48 h.

3.2.2.13 Measurement of lymphocyte proliferation by means of CFDA-SE

Since the CFSE membrane fluorescence dye is transferred to daughter cells in the same proportion, a decrease in fluorescence intensity can be followed (as cells multiply). CFSE is therefore widely used as an indirect proliferation marker. Unstained cells were used as controls.

3.2.3 Studies using the Rcho-1 trophoblast cell line

3.2.3.1 Cell culture of the Rcho-1 trophoblast cell line

Rcho-1 trophoblast cells represent a stem cell population capable of differentiation along the trophoblast giant cell lineage (Faria and Soares, 1991). The cells can be manipulated to proliferate or differentiate depending upon culture conditions (Peters *et al.* in: Methods in Molecular Biology, 2000). This cell line was a kind gift of Dr. Michael Soares, and was first established in his laboratory from a rat choriocarcinoma.

Cells were cultured in proliferation medium consisting of RPMI containing L-glutamine supplemented with 20% FBS (Cambrex), 1 mM sodium piruvate, 100 μg/ml penicillin, 100 μg/ml streptomycin and 50 μM β-mercaptoethanol. Cells were grown at a density of 1-2.10^6 cells/T 75 cm^2 flask. Cells were routinely maintained at subconfluent conditions and fed at a 2-day interval. It is very important to maintain the cells at subconfluent conditions, since high cell density facilitate trophoblast giant cell formation.

For differentiation of the Rcho-1 trophoblast stem cells into trophoblast giant cells, FBS was replaced by horse serum. Since a 1 to 10% of horse serum was mentioned in the literature, different concentrations of horse serum were tested. Finally, cells were differentiated using 10% of horse serum since it showed the best differentiation. The differentiation process takes normally place during 7 days, period after which the cells are fully differentiated.

3.2.3.2 Treatment of the Rcho-1 cells with CoPPIX and ZnPPIX

In order to determine the best concentration of protoporphyrin necessary to cause a diminution or augmentation of HO-1 protein expression in Rcho-1 cells, different concentrations of CoPPIX and ZnPPIX were tested, namely 25, 50, 100 and 200 μM. It was found that 50 and 100 μM of ZnPPIX were able to down-regulate the expression of HO-1, as measured by

Western Blot. CoPPIX did not induce a down- or up-regulation of HO-1 in these cells. Nevertheless, CoPPIX was used as a control for the use of ZnPPIX, at the same concentrations in order to assess whether the effects of ZnPPIX observed in the cells is due to the down-regulation of HO-1 or to the toxic effect of the porphyrin.

Two approaches using CoPPIX and ZnPPIX were performed. First, the porphyrins were employed to test the effect of a down-regulation of HO-1 in undifferentiated stem cells. In this case, cell viability was assessed by trypan blue staining.

In a second approach, CoPPIX or ZnPPIX were used during the whole differentiation process of the cells, changing the media when necessary (normally every two days) in order to investigate the influence of HO-1 down- or up-regulation in the differentiation from stem cells into giant cells. A control of differentiation using differentiation media without CoPPIX or ZnPPIX was always included.

3.2.3.2.1 Protein isolation and Western blot analysis

For protein isolation, cells were trypsinized as usual and then washed with PBS. For protein isolation, cells were lysed in a protein isolation buffer containing HEPES, CHAPS and DTT. After isolation, homogenates were centrifuged for 10 min at 10.000 g, and the supernatant containing the proteins was transferred to a fresh tube. Protein concentration was assessed using the BioRad Protein Assay as indicated by the manufacturer. Protein samples were kept at -80°C and when working with them, they were always kept on ice.

For Western Blot analysis, 15 µg of protein were transferred into a 10% polyacrilamide gel and a SDS-PAGE in denaturizing conditions was performed at 120V using a Mini-Protean 3 electrophoresis gel system. Then, proteins were transferred into a nitrocellulose membrane using a Mini-trans-Blot system for 1h at 180 mA. After the transfer, efficient protein transfer was assessed by staining of the membrane of Ponceau Red. The expression of HO-1 was analyzed (in the membrane) by immunoblot as follows: the membrane was blocked with 5% milk powder in TBS O.N. at 4°C, and further washed with TBST, 3 x 5 min each time. The first antibody (rabbit anti-HO-1 polyclonal antibody, diluted 1:500 in 5% milk in TBS) was applied for 2 h at R.T. After washing with TBST, the samples were incubated with the secondary antibody (anti-rabbit horseradish peroxidase, diluted 1:1000 in 5% milk in TBS) for 1 h at RT. The membrane was washed again, and the reaction was finally developed by using chemoluminiscence (ECL Assay of Amersham) and exposed onto Kodak Miomax MR Imaging film.

The intensity of the bands was quantified by using the Quantity One® Software, Version 4.5.2 from Bio-Rad.

3.2.3.2.2 Treatment of glass slides with poly-L-lysine

In order to be able to analyze the phenotype of Rcho-1 cells by immunohistology, the cells were grown on glass slides previously treated with poly-L-lysine (Sigma). Poly-Lysine enhances electrostatic interaction between negatively-charged ions of the cell membrane and positively-charged surface ions of attachment factors on the culture surface. When adsorbed to the culture surface, it increases the number of positively charged sites available for cell binding. Briefly, slides were incubated in sterility for 5 min with a 0.01% solution of poly-L-lysine at RT, then washed with sterile distilled water and allowed to dry for at least 2 hours. Covered slides were kept under sterility until use, and the cells have shown normal growth patterns on these slides.

3.2.4 Studies using mice deficient in *Hmox-1*

The fact that a homozygous mating of *Hmox1* deficient mice does not yield progeny (Poss and Tonegawa, 1997; Yet *et al.*, 1999), and that the mating of heterozygous mice does not yield the expected Mendelian rate suggests that HO-1 plays a very important role in pregnancy. For this part of the work, animals partially or totally deficient in *Hmox1* in a BALB/c genetic background- first established in the lab of Dr. Lee by Shaw-Fang Yet (Yet *et al.*, 1999) were used as a part of a cooperation project with Prof. Miguel Soares, from the Instituto Gulbenkian de Ciencia in Oeiras, Portugal, who provided us with the mice, after MTA agreement with Dr. Yet from Harvard. This part of the work was divided in different parts, which are explained below.

3.2.4.1 Influence of the partial or total loss of *Hmox1* in the pregnancy outcome

For this, different mating combinations of females and males partially or totally deficient in *Hmox1*, as well as of wild type mice were performed. A schematic representation of these mating combinations is shown in Fig. 18.

This was performed in a syngeneic (BALB/c x BALB/c) and allogeneic (C57/BL6 x BALB/c) background. A schematic representation of the groups analyzed is shown in Fig. 18.

Part of the mating combinations as well as the analysis of the abortion rates and collection of the samples of some of the animals was performed by Ivonne Wollenberg and Prof. Dr. Ana

Claudia Zenclussen from our group at the Instituto Gulbenkian de Ciencia. This work was continued in our laboratories by me and the *Hmox1*$^{-/-}$ colony is currently being maintained at the animal facility of Virchow Klinikum, Charité.

3.2.4.1.1 DNA isolation from fetuses and resorptions

The isolation of DNA from the fetuses and resorptions was performed using the peqGOLD Tissue DNA Mini Kit. For that, fetuses and resorptions were previously washed with PBS in order to remove the rests of blood from the mother, and the samples were grinded in a mortar using a pistil under the influence of liquid nitrogen. The samples were taken up in TL Buffer, and after adding OB™ protease and were further incubated at 55°C in a shaking water bath for at least three hours. After that, isolation was performed following manufacturer´s instructions, using HiBind® DNA columns for the specific bindng of DNA. After two washing steps, samples were eluted with elution buffer and kept at -20°C until further use.

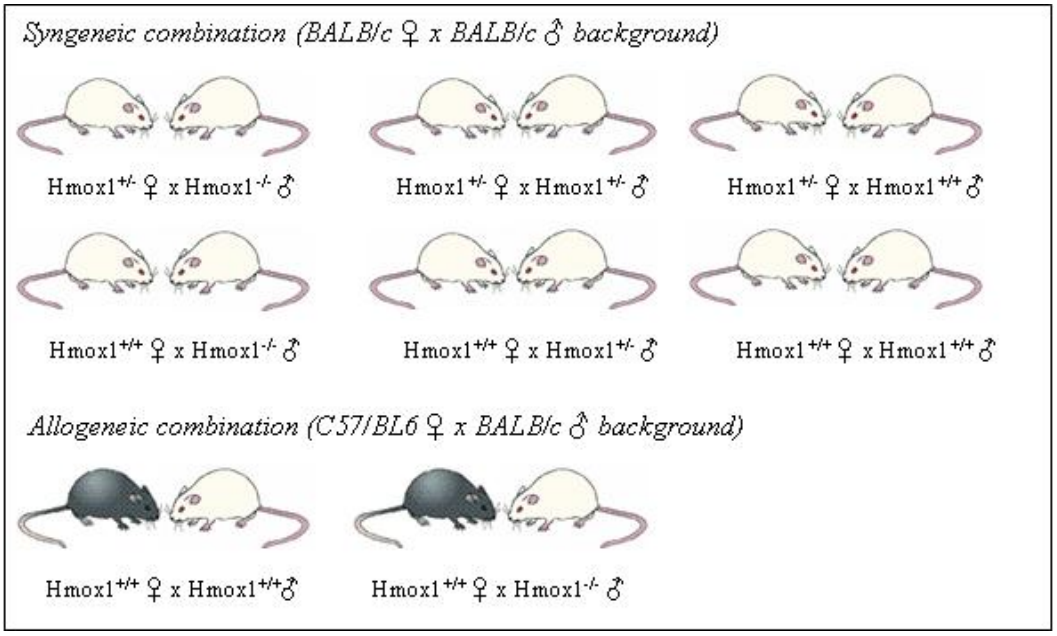


Fig. 18 : Experimental setting.

Heterozygous females for Hmox1 as well as wild type females were mated with knockout, heterozygous or wild type BALB/c males. For the syngeneic combination, BALB/c females were mated to BALB/c males, whereas for the allogeneic combination C57BL/6 wild type females were mated with BALB/c males knockout or wild type for Hmox1.

3.2.4.1.2 Genotyping of the fetuses and resorptions

The fundament for the PCR reaction used for genotyping of the fetuses and resorptions is based in the way that the knockouts were generated, which is schematized in Fig. 19. The mutated gene lacks the E3 region of the *Hmox1* gene, and possesses a Neo resistance gene instead. As it can be deduced from this Fig. 19, knockout fetuses show amplification only with the HO/E4-Neo1 primers which amplifies a 400-bp fragment of the mutated allele, whereas wild type fetuses will show amplification only with the HO/E3-HO/I3R set of primers, which amplifies a 456-bp fragment of the wild type allele. Accordingly, heterozygous fetuses show amplification with both set of primers. Table 6 shows the sequences of the primers used for the PCR reaction.

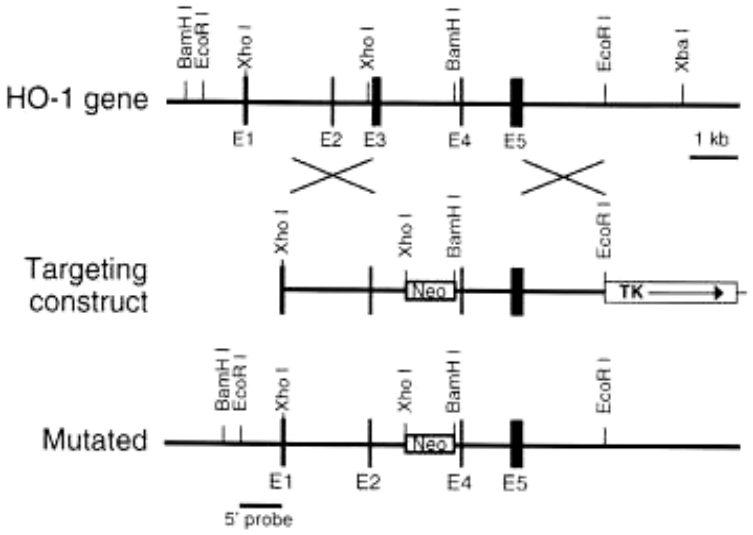


Fig. 19 : Deletion in the Hmox1 gene for the generation of the knockout colony (by Yet *et al.*, 1999).

The mutated gene lacks the E3 region and possesses a gene of resistance to Neomycin (Neo).

Fetuses and resorptions were genotyped using the standard procedure used for genotype the colony. This was done by means of traditional PCR, which was optimized for the GoTaq® DNA Polymerase (Promega) using 2.5 units of polymerase with Green GoTaq® Buffer containing 1.5 mM $MgCl_2$ (final concentration). This buffer allows the direct transfer of the sample into agarose gel for visualization with ethidium bromide. The reaction mixture was completed with 25 pmol of each primer (forward and reverse) and completed with water to a final volume of 50 µl.

The PCRs for the HO/E3-HO/I3R and for the Neo1-HO/E4 set of primers were performed in different tubes, making duplicates for each sample. Every time that PCR was performed, wild type, knockout and heterozygous controls were included. The PCR was performed by 3 cycles of 5 min at 94°C followed by 30 s at 58°C and 30 s at 72°C. This was followed by 25 cycles at 94°C, 58°C and 72°C, for 30 s each, followed by a final elongation step of 7 min at 72°C. Samples were transferred into an agarose gel and visualized using ethidium bromide.

Table 6 : Sequences of the primers used for genotyping of fetuses and resorptions

Primer	***Sequence***
Neo1	5´-TCT TGA CGA GTT CTT CTG AG-3´
HO/E4	5´-ACG AAG TGA CGC CAT CTG T-3´
HO/E3	5´-GGT GAC AGA AGA GGC TAA G-3´
HO/I3R	5´-CTG TAA CTC CAC CTC CAA C-3´

The Neo1-HO/E4 set of primers amplifies a 400-bp fragment of the mutated allele, whereas the HO/E3-HO/I3R set amplifies a 456-bp fragment from the wild type allele (primer sequences first described in Fujita et al., 2001).

3.2.4.2 *In vitro* fertilization

In vitro fertilization (IVF) was carried out during my research period at the Instituto Gulbenkian de Ciencia, Oeiras, Portugal, according to the host laboratory´s procedure and with the assistance of Sofia Rebelo, an expert in the field. It included the following steps: induction of superovulation by hormonal treatment, preparation of the dishes for IVF, sperm collection, collection of the oviducts, fertilization, washing of the oocytes, and assessment of the fertilization rates, which will be briefly described below:

3.2.4.2.1 Superovulation

For superovulation, 2-3 months old females were intraperitoneally injected with 5.0 IU of PMSG (Pregnant Mare Serum Gonadotropin), followed by an injection of 5.0 IU of hCG (human chorionic gonadotropin) 48 h later.

3.2.4.2.2 Preparation of the dishes for IVF

A small Petri dish with one drop (500 µl) of HTF medium in the middle of the dish was prepared ("sperm dish"). This drop was covered with oil and placed O.N. at 37°C. The "fertilization dish" (1 per three females) consisted in a 60 mm Petri dish with 500 µl of HTF medium covered with oil. This dish was also incubated O.N. at 37°C. One "wash dish" per each fertilization dish was prepared using 4 drops x 125 µl of HTF in a small Petri dish, covered with oil, which was also incubated O.N. at 37°C.

3.2.4.2.3 Sperm collection

For sperm collection, males were sacrificed by cervical dislocation 12 h after hCG was injected to the females. The *cauda epididymides* and *vasa deferentia* were dissected out and placed in the sperm dish, inside the medium drop. The *cauda epididymis* was dissected out making 5 slashes with a 30-gauge needle on a syringe and the sperm was gently squeezed out from the *vasa deferentia* by using forceps. Sperm was collected 15 to 45 min before the females reach 13 h post hCG injection. After 10 min-1 h incubation of the sperm in a 37° C incubator in a dish containing HTF medium, 10 µl of sperm was transferred into each fertilization dish.

3.2.4.2.4 Collection of the oviducts and fertilization

For collection of the oocytes, females were sacrificed 13 to 14 h after hCG injection by cervical dislocation and oviducts were taken as shown in Fig. 20. The oocytes were collected from the swollen ampulla (Fig. 21) and were found in a cloud together with cumulus cells. This "cloud" was taken to the drop and put in contact with the sperm. This procedure had to be very quick and not to take more than 5 minutes per plate.

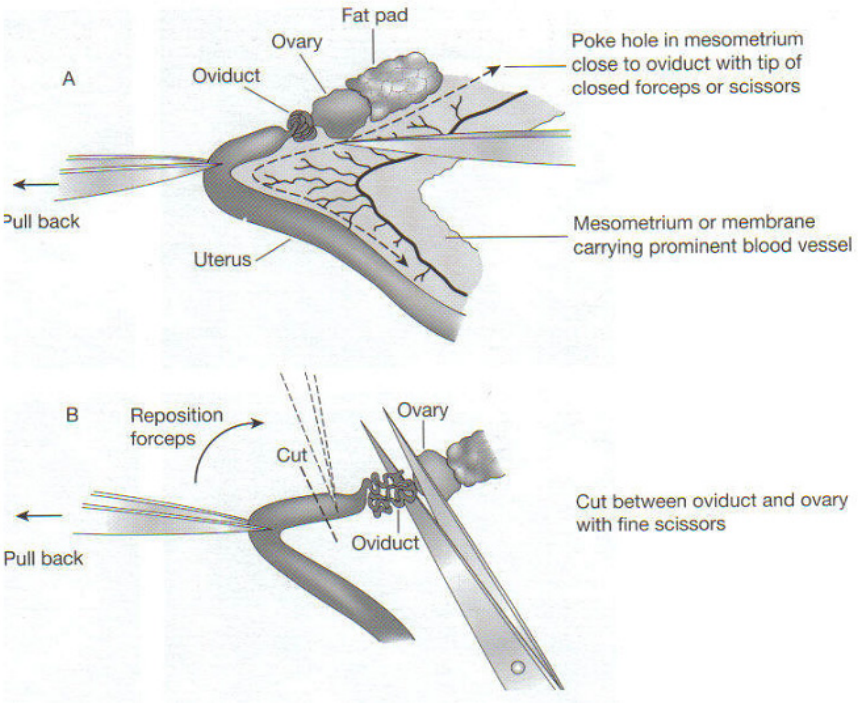


Fig. 20 : Dissection of the oviduct

Figure taken from Nagy et al. in: Manipulating the mouse embryo, 2003. A) The ovary, oviduct and the end of the uterus are separated from the mesometrium. B) A cut is made between the oviduct and the ovary. A second cut separated the oviduct from the uterus.

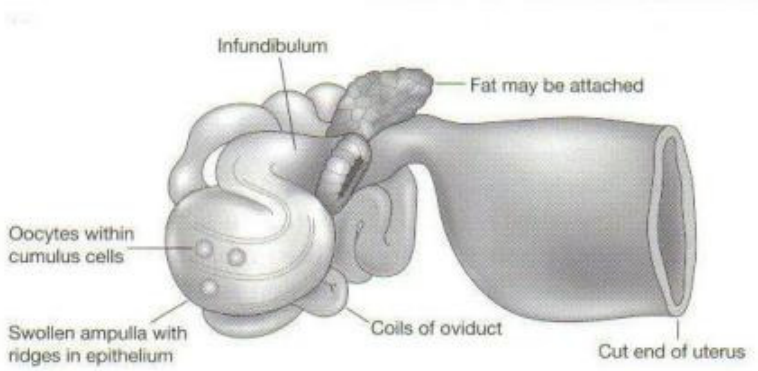


Fig. 21 : Isolation of oocytes from dissected oviduct

When the oviduct is removed soon after hormonal treatment, the oocytes surrounded by cumulus cells in the upper part of the oviduct (ampulla), and can be released by tearing the ampulla with fine forceps.

3.2.4.2.5 Washing of the oocytes

The manipulation of the oocytes was always carried out with a mouth pipette assembly, consisting of an aspirator mouthpiece, tubing, and a glass Pasteur pipette previously pulled on a flame (to create a narrow opening). Oocytes were placed in the fertilization drop, which already contained the sperm. After 4 to 6 h of incubation, oocytes were washed in order to remove the excess of sperm, and further incubated O.N. at 37°C. In this step, the number of total oocytes was counted and will be referred as one-cell stage.

3.2.4.3 Assessment of the fertilization rate

Two-cell stage was evaluated in order to address fertilization efficiency. The number of cells in two cell stage was determined under the microscope, and the fertilization rate was calculated according to the formula:

$$Fertilization\ rate\ (\%) = \frac{Number\ of\ oocytes\ in\ two\ cell\ stage}{Number\ of\ oocytes\ obtained} \times 100$$

Two-cell stage embryos were washed in M2 Medium, in order to remove the excess of HTF Medium.

3.2.4.4 Male vasectomisation

For the vasectomisation procedure, males were anesthetized and both testes were pushed down into the scrotal sac by applying pressure to the abdomen. A small incision was made through the skin along the midline of the scrotal sac. Testes were taken out one at a time, and the membrane of the testis suffered a small incision close to the vas deferens. The *vas deferens* was identified as a bright white tubule with a single blood vessel. Using forceps, both the *vas deferens* and the blood vessels were hold and cauterized in two positions with red hot tips, taking out a small portion of the *vas deferens*. Testes were put back in place, and the skin was clipped together. Males were kept on a heating pad until they recovered from the injected anesthetic, and were mated with the females after at least one week.

Vasectomized males (BALB/c background) were bred with females to produce pseudopregnant recipients for oviduct and uterine transfer.

3.2.4.5 Embryo transfer

The surgical procedure of embryo transfer was performed by Sofia Rebelo, member of the Instituto Gulbenkian de Ciencia, an expert on the field. Two-cell stage embryos were transferred into female recipient mice that have been previously mated with vasectomized males and which exhibited plug in the morning of the transfer. In order to assure that females will show plug, recipient females were injected with hormones and mated with the males immediately after HCG injection. After mating with the vasectomised (sterile) male, the female´s reproductive tract is supposed to become receptive for transferred embryos, even though their own unfertilized oocytes degenerate. The day of the embryo transfer was considered as day 0 of pregnancy.

The transfer was performed anesthetizing the female and making a small incision in the skin along the dorsal midline, at the level below the last rib. As shown in Fig.22, the ovarian fat pad, attached to the ovary, oviduct and uterus was pulled out in order to make the transfer into the oviduct (infundibulum). Embryos in two-cell stage in M2 Medium were transferred into the oviduct using a transfer pipette (mouth-pipetting device). Once transferred in both oviducts, the uterus, oviducts and ovaries were put back inside the body cavity and the skin was closed with wound clips. Each side of the uterus received 10 embryos in two-cell stage.

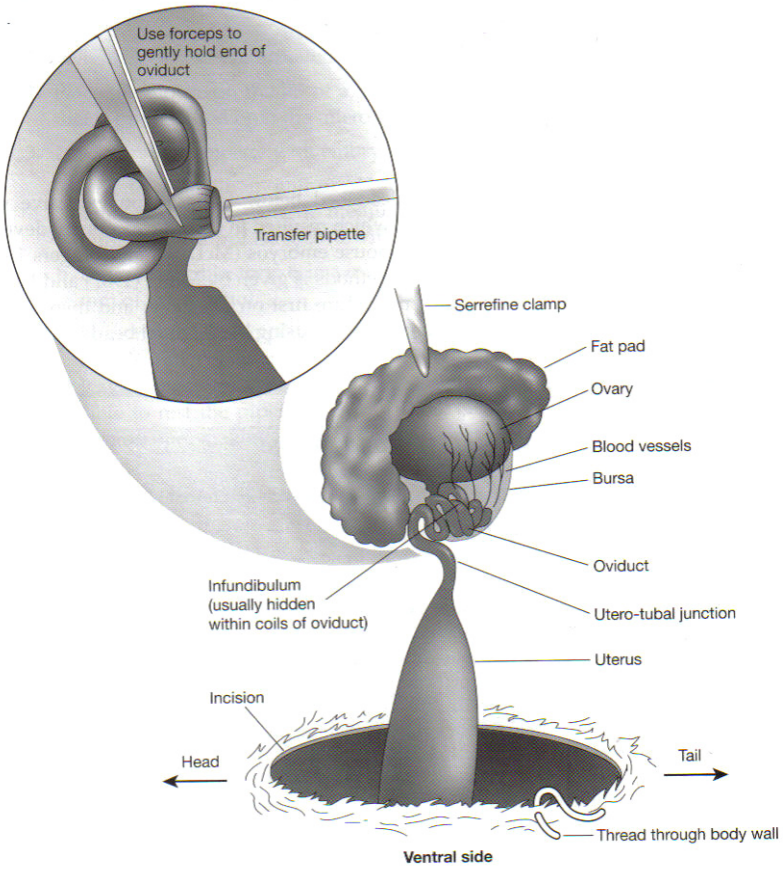


Fig. 22 : Embryo transfer into the oviduct

Fig. taken from Nagy et al. in: Manipulating the mouse embryo, 2003.

3.2.4.5.1 Assessment of the pregnancy success

On day 14 of pregnancy, females were sacrificed, the uteri removed, and the implantation sites were documented. Resorptions were kept for further analysis, as well as spleen, liver, thymus, ovaries and decidua. Abortion rates were calculated as the ratio of abortion sites to total implantation sites as previously described.

3.2.4.6 Analysis of ovarian follicle development

Ovaries from *Hmox-1*$^{+/+}$ and *Hmox-1*$^{-/-}$ females used as donors were embedded in paraffin as previously described in 4.2.1.9. Samples were cut in 5 µm slides and stained with Hematoxilin and Eosin as previously described in 4.2.1.11. The analysis of the follicles was carried out under light microscope, counting the follicles in each stage of maturation (primordial, primary, secondary and mature) using a 20X magnification. Both ovaries of each animal were analyzed, counting at least three slides per ovary.

3.2.5 Data analysis and statistics

Most of the data presented in this thesis is represented by boxplots. This type of graphic was chosen since it is the best way to present data that is analyzed by non-parametric tests.

An example of a boxplot representation is shown in Fig. 23. The boxes show the lower quartile (25th percentile), the median and the upper quartile (75th percentile). The difference between the upper and lower quartiles is the inter-quartile range (IQR) and it contains 50% of the sample. The smallest and largest values are indicated by the small horizontal bars at the end of the whiskers. Outliers (defined as 1.5-fold the IQR, Henderson, 2006) are normally shown as circles and/or asterisks below or above boxplots.

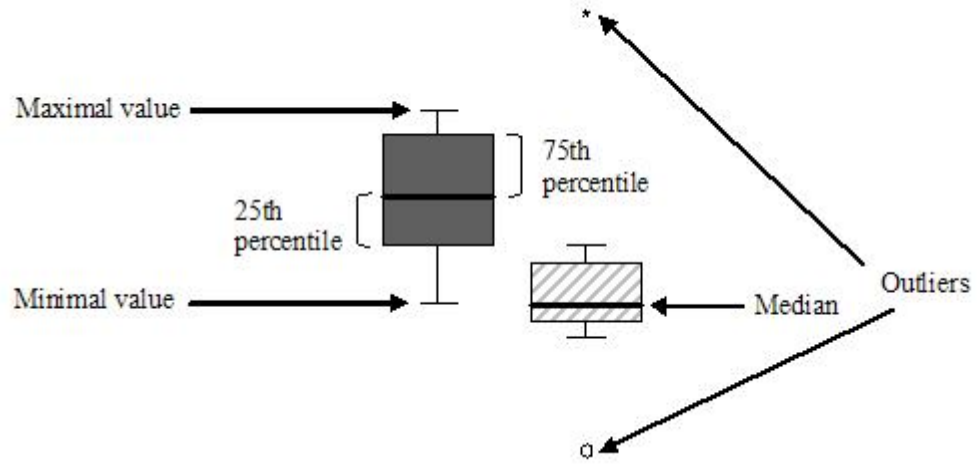


Fig. 23 : Representation of the data with boxplots

Boxplots were used as a representation for non-parametric data, showing between the maximal and minimal value the 100% of the data obtained.

Data from the study using the murine model of abortion are presented as medians or medians ± 75% quartiles. Analysis and graphics were made with the SPSS 11.5 software. When appropriate, statistical analysis of the data was performed using the non-parametric Kruskal-Wallis test followed by Mann-Whitney *U*-test. In all cases, $p<0.05$ was considered a statistically significant difference.

Data obtained using the Hmox1 deficient mice are presented as mean ± SD when analyzing the *in vitro* fertilization results. The statistical analysis of the *in vitro* fertilization was performed with the total number of oocytes, fertilized and unfertilized, using the Fisher´s exact

test. The graphics and statistics of this part of the work were obtained with the GraphPad Prism 4 Software. The data regarding follicle development is shown as median ± interquartile range and were analyzed by the Mann-Whitney *U*-test.

4 Results

4.1 Morphology of murine implantations (healthy fetuses and resorptions)

Immunological rejection during pregnancy is characterized in the mouse by necrotic tissue and infiltration of immune cells at the site of implantation. This site is usually called "resorption" and resembles human abortion (or miscarriage). Fig. 24 depicts a typical abortion event, near to a healthy fetus at its implantation site. As seen in the picture, resorptions are hemorrhagic necrotic areas of the tissue that are considerable smaller than the normal placentas and their fetuses.

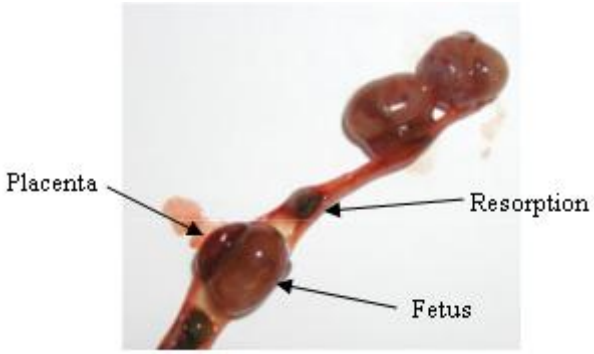


Fig. 24 : Representative picture of the feto-maternal interface in a murine pregnancy

Resorptions and healthy fetuses can be clearly differentiated from each other due to the hemorrhagic and necrotic appearance of the resorption, as well as due to its smaller size as compared to the healthy implantation site.

Hematoxilin-Eosin staining of healthy placental tissue made it possible to identify the different cell types present in a murine placenta on day 14 of gestation. Fig. 25 shows a typical placenta, staining in which all types of cells can be appreciated.

The outer layer of the placenta, even after dissection from the uterus, is contaminated with maternal decidual cells which usually appear as a thin layer of small cells (Nagy *et al.* in: Manipulating the mouse embryo, 2003). In the mature placenta, the spongiotrophoblast forms the middle layer of the placenta between the outermost giant cells and the innermost labyrinth. Finally, the last type of cells found in the placenta are the glycogen cells, which appear within the trophoblast layer (reviewed in Cross, 2005).

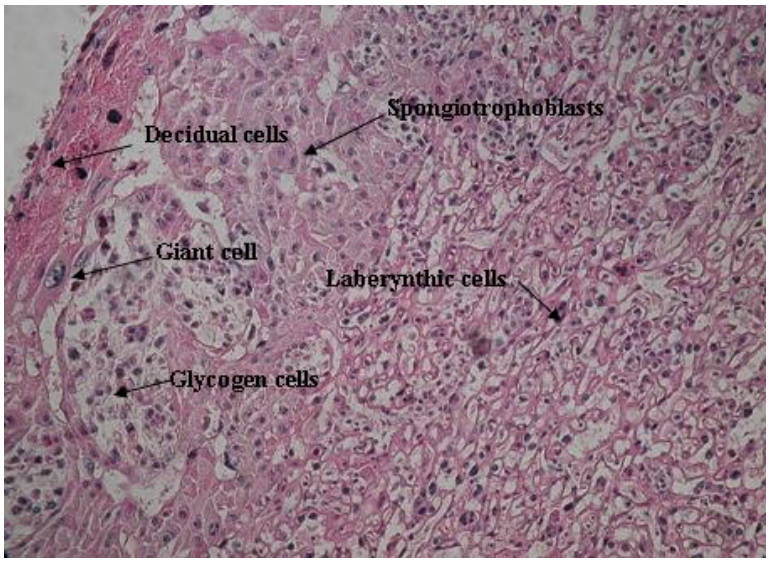


Fig. 25 : Representative HE staining showing the different cell types in a murine placenta

4.2 *In vivo* application of AdHO-1/GFP in a murine model of abortion

4.2.1 Effect on the pregnancy outcome

The application of an adenoviral vector containing HO-1 was employed in order to analyze the effect of a specific up-regulation of HO-1 on the pregnancy outcome of mice undergoing spontaneous immunological abortion.

As expected (Chaouat et al., 1995; Clark et al., 1997), DBA/2J-mated CBA/J mice presented significantly higher abortion rates when compared to the normal pregnant combination CBA/J x BALB/c. Interestingly, abortion-prone mice receiving 1.10^5 PFU AdHO-1/GFP showed a significant diminution in the abortion rate as compared to PBS-treated abortion-prone mice ($p<0.05$) or mice receiving 1.10^5 AdEGFP ($p<0.05$), pointing out a beneficial effect of HO-1 on pregnancy outcome (Fig. 26). Since mice receiving the adenoviral construct without HO-1 showed similar abortion rates as the abortion-prone group, it is to speculate that the injection of sole adenoviral particles -known to be immunogenic- did not significantly affect the pregnancy outcome (Fig. 26). However, a small augmentation in the abortion rate was observed in the group receiving the AdEGFP vector, indicating that injection of AdHO-1 had a beneficial effect despite the immunological response that the injection of adenoviruses implied.

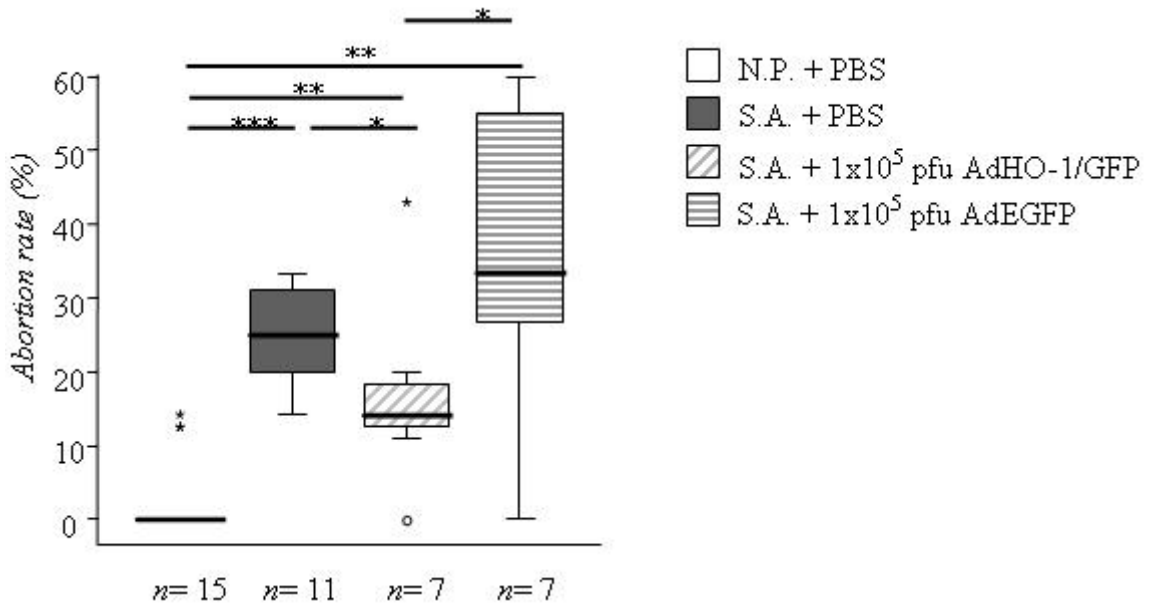


Fig. 26 : Specific up-regulation of HO-1 improves pregnancy outcome in a murine model of abortion

Data are represented by medians ± 75% quartiles. N.P.= Normal Pregnancy; S.A.= Spontaneous abortion. Circles and/or asterisks above box plots indicate outliers. Significant differences between groups are indicated as asterisks above lines. *p≤ 0.05, **p≤ 0.01 and *** p≤ 0.001, as analyzed by the Kruskall-Wallis non-parametric test between all groups, followed by the Mann-Whitney U-test for two particular groups.

Interestingly, abortion-prone mice receiving 1.10^8 PFU AdHO-1/GFP presented a slight and not significant diminution in the abortion rate (n = 8) when compared to mice receiving 1.10^8 AdEGFP (n = 7), suggesting that a too high HO-1 concentration is not pregnancy-protective. This data is shown as Table 14 in the appendix. These two groups will not be taken into account for the graphics, since its application did not show any effect on the pregnancy outcome. Nevertheless, the data regarding these two groups are shown in the appendix as Table 14.

Since the percentage of abortion rate depends on the number of implantations, the number of implantations was calculated for every group. As it can be observed in Fig. 27, the number of implantations was comparable between all groups (Fig. 27). Considering this, it can be stated that the up-regulation of HO-1 has beneficial effect of pregnancy outcome.

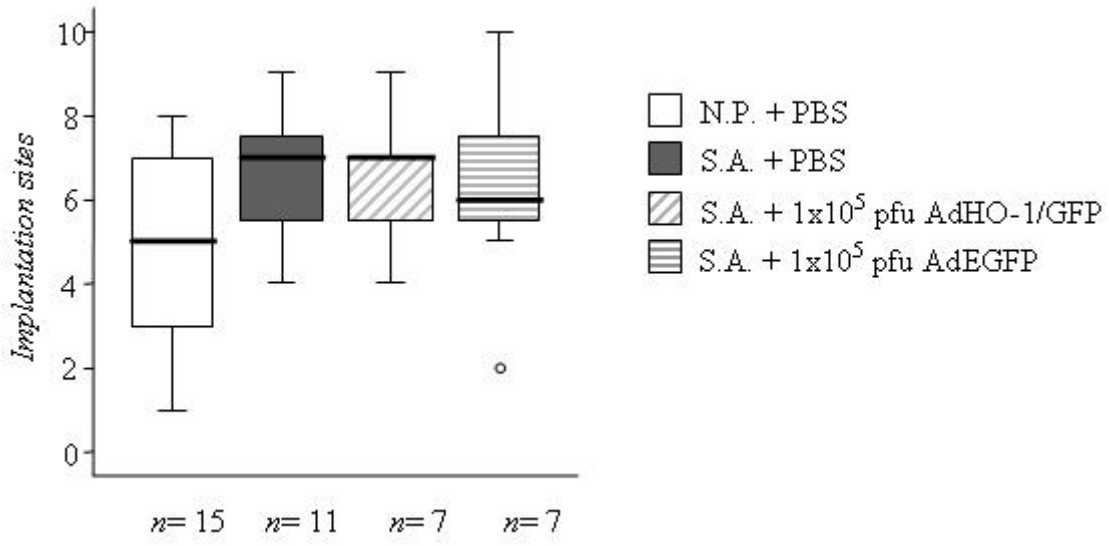


Fig. 27 : The number of implantation sites was similar between all experimental groups

Data are represented by medians ± 75% quartiles. N.P.= Normal Pregnancy; S.A.= Spontaneous abortion; Circles below box plots indicate outliers. No significant differences were found between the groups as analyzed by the Kruskall-Wallis non-parametric test between all groups, followed by the Mann-Whitney U-test for two particular groups.

4.2.2 Heme Oxygenase-1 expression at the feto-maternal interface

In order to analyze whether the effect of HO-1 up-regulation is noticeable in the levels of HO-1 at the feto-maternal interface 9 days after treatment, the expression of HO-1 was measured at mRNA and protein levels in placental tissue. Table 7 shows the mRNA levels of mouse HO-1 in all groups, where a slight augmentation of mouse HO-1 mRNA can be seen in the group receiving AdHO-1/GFP. However, this augmentation is not statistically significant. HO-1 mRNA levels where significantly augmented in placentas of mice receiving the higher doses of AdHO-1/GFP in comparison with mice receiving low doses (Table 17, Appendix).

By measuring mRNA of human HO-1, the one coded in the adenovirus used for the *in vivo* experiment, only few samples showed amplification curves and most of them did not show any amplification after 40 cycles. This may be due to the fact that the mice were killed on day 14 of pregnancy, 9 days after injection of the adenoviruses. This makes sense as it is reported that adenoviruses remain between 5 and 20 days post-infection (Dai *et al.*, 1995).

Table 7 : No significant differences in the placental HO-1 mRNA levels between the groups

	N.P (n=5)	*S.A. (n=8)*	*S.A. + AdHO-1/GFP (n=5)*	S.A. + AdEGFP (n=7)
Mean	0.05076	0.03590	0.04565	*0.05047*
SD	0.04651	0.01383	0.02169	*0.01575*
Median	*0.02906*	*0.02967*	*0.04404*	*0.04443*

mRNA levels of HO-1 normalized to b-actin. N.P.= Normal Pregnancy; S.A.= Spontaneous abortion. No significant differences were found between the groups as analyzed by the Kruskall-Wallis non-parametric test between all groups, followed by the Mann-Whitney U-test for two particular groups.

Protein levels of HO-1 were analysed by Western Blot using homogenates of whole placental tissues using b-actin as protein reference. Intensity of the bands was analyzed using the Quantity One Software (BioRad), and a HO-1/β-actin ratio was calculated for each sample. Fig. 28 shows an example of the β-actin and HO-1 Western Blot bands obtained.

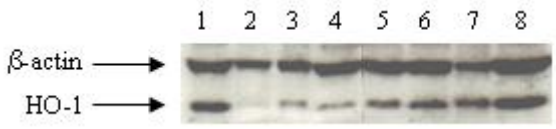


Fig. 28 : Example of the Western Blot from placental samples

1 and 5) CBA/J x BALB/c placentas

2 and 6) CBA/J x DBA/2J placentas

3 and 7) CBA/J x DBA/2J + AdHO-1/GFP placentas

4 and 8) CBA/J x DBA/2J + AdEGFP placentas

Bands were analyzed using a densitometer being the densitometric units for HO-1 normalized to those of b-actin. The results are showed in Table 8, showing mean, standard deviation and median for each analyzed group. No differences were obtained in HO-1 protein levels between the groups, as analyzed by the Kruskall Wallis test between all groups, as well as no differences between the groups, as analyzed by the Mann Whitney test. A trend towards a diminution of HO-1 expression in the abortion prone-group when compared to the normal pregnant group could be observed (Table 8), in accordance with previous results from our group (Zenclussen *et al.*, 2005). A tendency towards an augmentation was observed in the group receiving AdHO-1/GFP as well as in the group receiving AdEGFP (Table 8).

Table 8 : Placental protein levels of HO-1 were similar between all groups at the analyzed time point

	N.P. (n=5)	*S.A. (n=5)*	*S.A. + AdHO-1/GFP (n=4)*	S.A. + AdEGFP (n=3)
Mean	0.3188	0.1370	0.3924	*0.3681*
SD	0.2296	0.1524	0.2978	*0.2267*
Median	*0.3165*	*0.05028*	*0.2772*	*0.4157*

Protein levels of HO-1 as normalized to β-actin, N.P.= Normal Pregnancy; S.A.= Spontaneous abortion. No significant differences were found between the groups as analyzed by the Kruskall-Wallis non-parametric test between all groups.

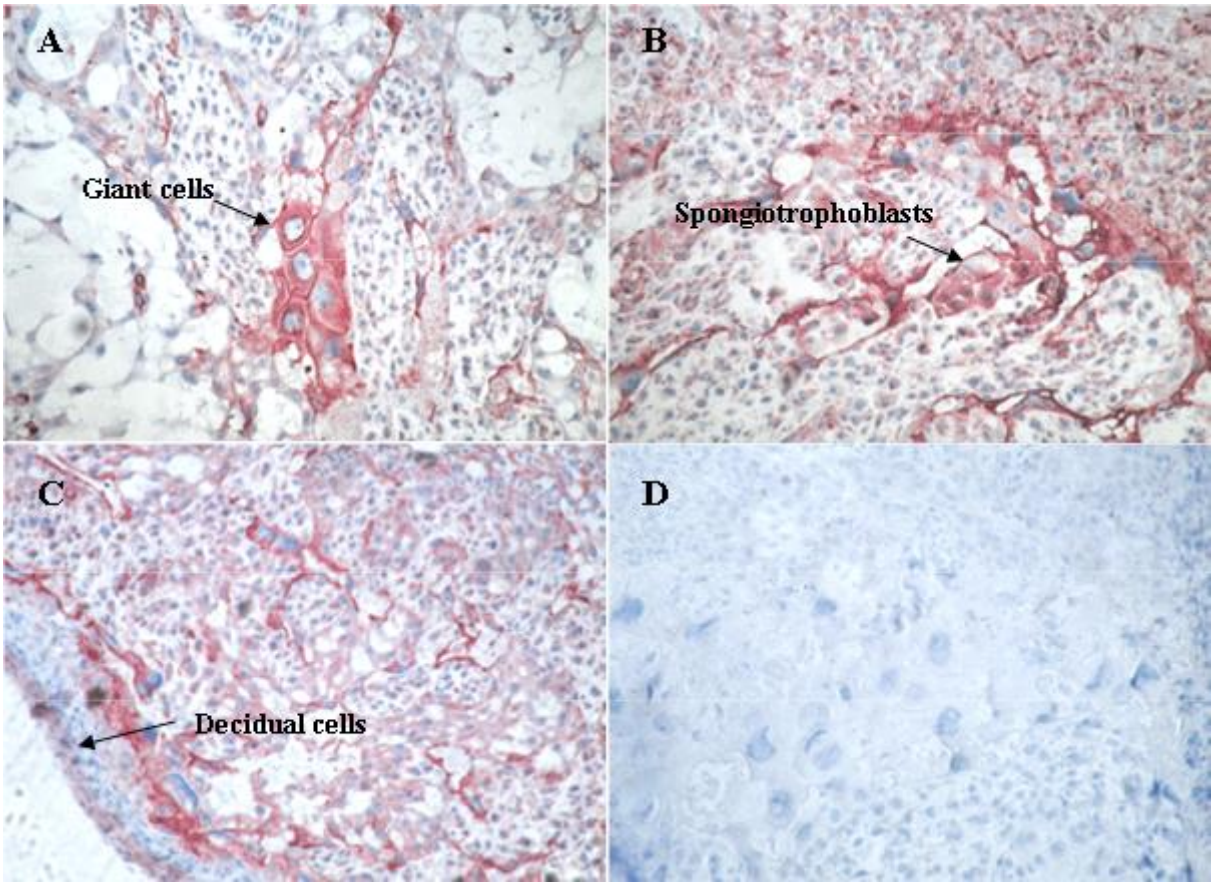


Fig. 29 : HO-1 is highly expressed in all murine placental cell types as well as in the decidua as analyzed by immuno-histochemistry in paraffin-embedded tissues.

Immunohistochemical analysis of HO-1 in paraffin-embebbed tissues. A) shows intense HO-1 staining on giant cells; B) depicts HO-1 expression on spongiotrophoblasts. C) HO-1 expression was also found on decidual cells. D) represents the negative control of the staining. All pictures were taken using a 20X magnification of the objective lens.

Protein levels of HO-1 were also measured by immunohistochemistry in order to analyze the HO-1 expression in each different cell type and have more detailed information regarding local HO-1 expression. HO-2 expression was as well analyzed by immunohistochemistry.

Again, no differences were observed between the groups for murine HO-1, and also no differences were found for HO-2 expression. Representative pictures for HO-1 staining are shown in Figures 29A-C, were it can be seen that HO-1 is highly expressed throughout the placenta as well as in decidua, with its highest expression in giant cells. Since giant cells are very important for the onset of placentation, it is tempting to assume that HO-1 may play an important role in placentation. Fig. 29D shows the negative control of the staining. HO-2 expression was found throughout the whole placenta, with similar patterns of expression as for HO-1.

4.2.3 Detection of adenoviral particles in placental and fetal tissue

Although no differences were observed in the levels of HO-1 between the groups, it was possible to detect human HO-1 mRNA (derived from the vector) in some placenta samples from animals treated with Ad-HO-1**.** This suggests that the adenovirus reached the feto-maternal interface. The use of a therapeutic vector containing GFP made possible the analysis of the presence of the construct in the reproductive tract to confirm that the applied adenoviruses reached the feto-maternal interface. GFP expression was analyzed by fluorescence microscopy as well as by real-time PCR.

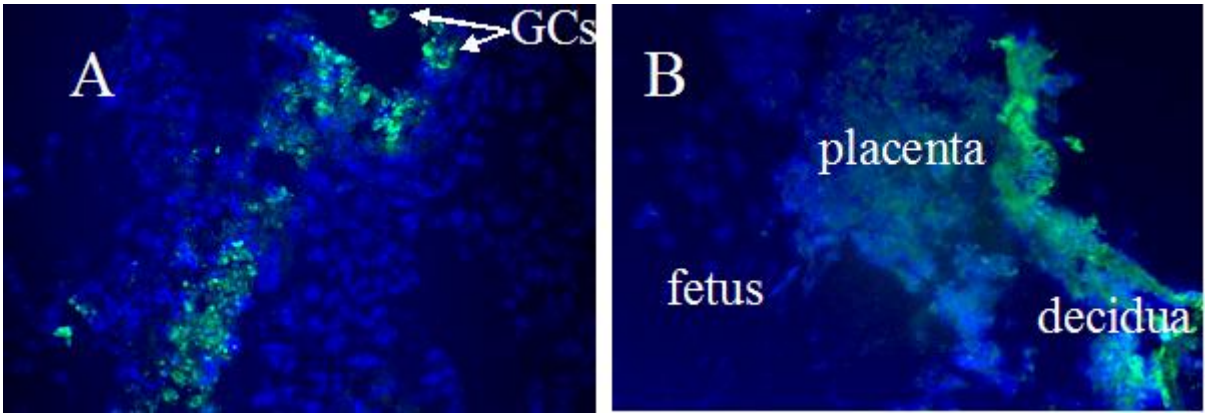


Fig. 30 : GFP$^+$ cells were found in the placenta and not in the fetus when analyzed by fluorescence microscopy

A) shows GFP+cells at the feto-maternal interface from abortion-prone mice receiving 1 x 10^5 PFU AdHO-1/GFP; GCs = giant cells; B) depicts GFP+ cells in decidual and placental cells but not in the fetus after the transfer of 1 x 10^5 PFU AdEGFP. Pictures were taken in a 20X magnification of the objective lens.

GFP protein was in fact expressed at the feto-maternal interface in decidual cells as well as in giant cells, i.e. in the outer layer of the fetal-maternal interface as analyzed by fluorescent microscopy. Figure 30A shows a representative field of GFP^+ cells found in cryo-sections of placental tissue. Figure 30B shows positive GFP expression in decidual and placental tissue, while no expression could be observed in fetal tissue.

Further, a semi-quantitative measurement of adenoviral particles for GFP was performed by real time PCR in liver and placental tissues as well as in fetus homogenates in order to determine the tissue distribution of the adenoviral particles after injection. The higher GFP expression was found in maternal liver, which was expected considering the high hepatic tropism of adenovirus if applied intra-peritoneally (Huard *et al.*, 1995). A lower adenovirus expression was found in placental tissue, which confirms the GFP detection by fluorescent microscopy, and also very low levels of adenoviral particles could be found in fetus homogenates (Fig.31). Low adenovirus expression in fetuses after gene therapy was already described by other authors (Senoo *et al*., 2000). Because we homogenized fetal tissues using liquid N_2, the quantified virus may represent only the virus present in the fetal skin as already reported by other groups (Senoo *et al*., 2000), since effective gene transfer into the fetal organs e.g. liver or kidney is only possible when applying the vector into the umbilical vein (Senoo *et al*., 2000). However, this GFP may be not expressed at the fetal tissue, as no GFP expression could be observed in the fetuses by fluorescence microscopy.

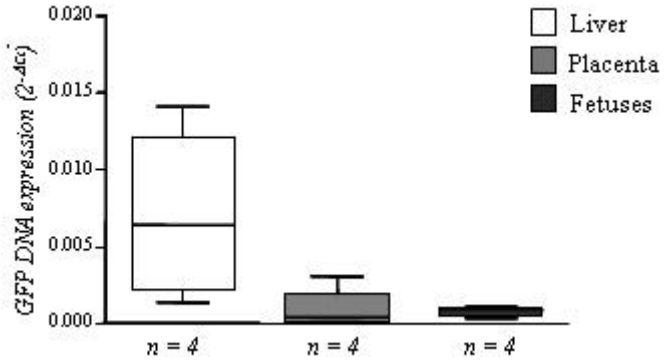


Fig. 31 : GFP DNA was mostly found in maternal liver after i.p. injection of AdEGFP as measured by real time PCR

GFP expression (relative to ApoB) measured with real time PCR in mice receiving $1x10^5$ PFU AdEGFP. The highest GFP expression was found in the maternal liver. Placentas and fetuses showed very low GFP expression.

4.2.4 *Ex-vivo* intracellular cytokine production by spleen and decidual lymphocytes as measured by flow cytometry

As HO-1 was previously described to affect the cytokine balance in transplantation models (reviewed in Katori *et al.*, 2002) and considering that a proper Th1/Th2 balance is important for successful pregnancy, systemic and local cytokine expression by T cells was measured by flow cytometry. Spleen and decidual cells were stimulated by PMA/Ionomycin, process which is known to stimulate mainly memory T cells.

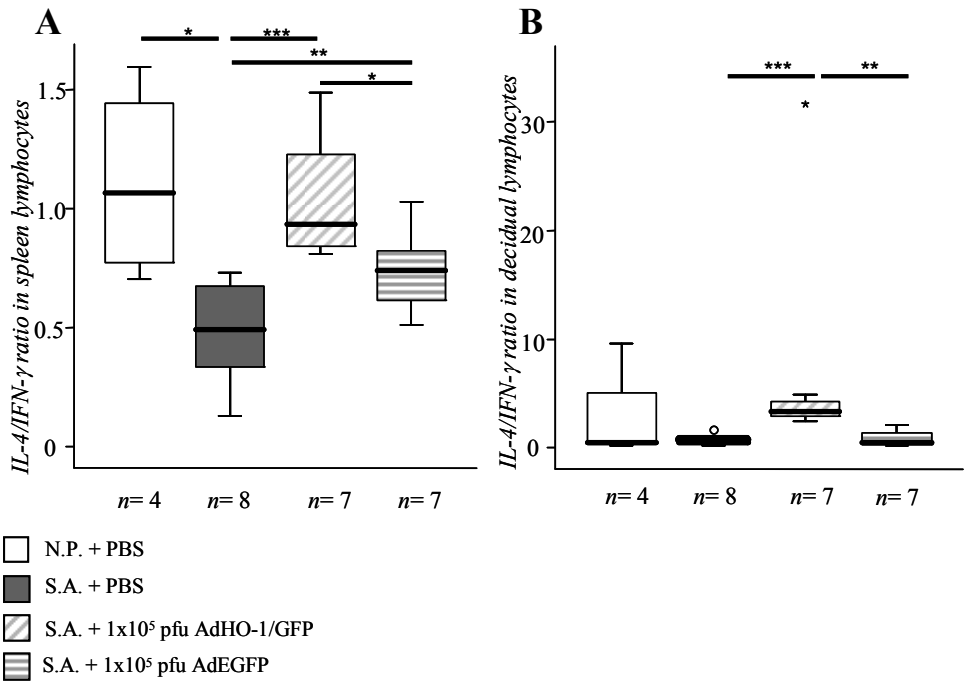


Fig. 32 : HO-1 up-regulation could significantly diminish the IL-4/IFN-γ ratio in spleen and decidual lymphocytes as analyzed by flow cytometry

Th1/Th2 ratios in spleen (A) or decidual lymphocytes (B) measured by the production of IFN-γ and IL-4 by flow cytometry after PMA/Ionomycin incubation in the presence of monensin. Data are represented by medians ± 75% quartiles. N.P.= Normal Pregnancy; S.A.= Spontaneous abortion; Circles and/or asterisks above box plots indicate outliers. Significant differences between groups are indicated as asterisks above lines. *p≤ 0.05, **p≤ 0.01 and *** p≤ 0.001, as analyzed by the Kruskall-Wallis non-parametric test between all groups, followed by the Mann-Whitney U-test for two particular groups.

T cells were gated according to their size and granularity, and the ability of Th2 (IL-4 and IL-10) and Th1 (IFN-γ and TNF-α) production was analyzed by flow cytometry. The results

were expressed as IL-4/IFN-γ or IL-10/TNF-α ratio, as it can be observed in Fig 32 for spleen (A) and decidual (B) lymphocytes.

Interestingly, a diminution in the IL-4/IFN-γ ratio could be observed in both, spleen and decidual lymphocytes from abortion mice when compared to normal pregnant mice, nicely supporting the Th1/Th2 paradigm (Fig. 32 A and B). Results are shown in Fig.32A and B, for spleen and decidual lymphocytes, respectively. Very interestingly, only mice treated with $1x10^5$ PFU but not with $1x10^8$ PFU AdHO-1/GFP showed a significant augmentation in the IL-4/IFN-γ cytokines ratio in both, spleen and decidual lymphocytes. Moreover, the IL-4/IFN-γ levels in this group were comparable to those obtained in the normal pregnancy group. High doses of HO-1 were not able to up regulate the IL-4/IFN-γ ratio, suggesting again that too much HO-1 is not pregnancy compatible.

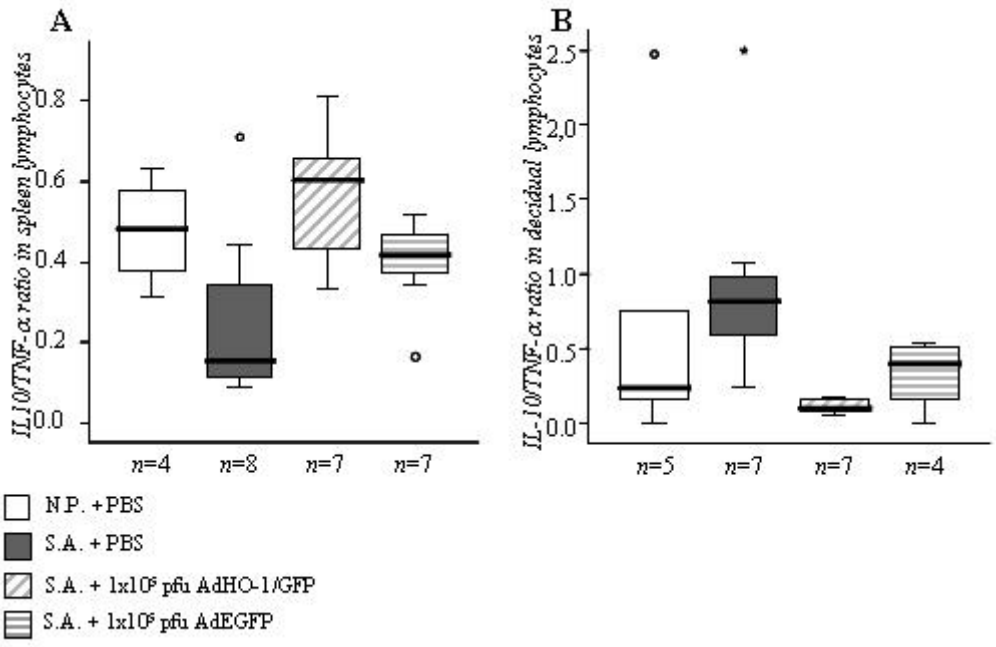


Fig. 33 : IL-10/TNF-α ratio in spleen and decidual lymphocytes as analyzed by flow cytometry

Th1/Th2 ratios in spleen (A) or decidual lymphocytes (B) measured by the production of TNF-α and IL-10 by flow cytometry after PMA/Ionomycin incubation in the presence of monensin. Data are represented by medians ± 75% quartiles. N.P.= Normal Pregnancy; S.A.= Spontaneous abortion; Circles and/or asterisks above box plots indicate outliers. No significant differences were obtained between groups as analyzed by the Kruskall-Wallis non-parametric test between all groups.

As depicted in Fig. 33A, a diminution in the IL-10/TNF-α ratio in spleen cells was found in abortion-prone animals compared to normal pregnant animals. HO-1 treatment up-regulated IL-10/TNF-α ratio when compared to abortion-prone mice. Nevertheless, this difference was not significant. Surprisingly, the levels of IL-10/TNF-α in decidual cells were augmented in abortion-prone mice when compared to normal-pregnant mice. Mice receiving AdHO-1/GFP as well as mice receiving AdEGFP showed similar IL-10/TNF-α levels as those obtained for the normal pregnant combination. Kruskall-Wallis analysis between all groups show no significant differences between all groups analysed (Fig.33B).

Medians of the cytokine levels for each measured cytokine are shown as a Table in the Appendix (Tables 15 and 16).

4.2.5 Lymphocyte infiltration at the feto-maternal interface

Since a Th2 shift in the cytokine production was observed in the AdHO-1/GFP treated group, it was interesting to analyze whether this treatment also provokes an augmentation in the number of lymphocytes reaching the feto-maternal interface. The marker CD3, expressed in all T cells, was analyzed at mRNA and protein levels.

Levels of CD3 mRNA were measured by real time RT-PCR in placentas (tissue of fetal origin) as well as in decidua (maternal tissue). As can be observed in Table 9, the CD3 mRNA levels were very low, confirming low amounts of the immune cells at the placenta. Besides, no major differences were obtained regarding CD3 expression in placental tissue between the groups. This suggests that the lymphocytes present in the placenta are prone to produce more Th2 and less Th1 cytokines after HO-1 treatment.

In decidual tissue (of maternal origin) CD3 expression was augmented in animals receiving AdHO-1/GFP. As can be seen in Fig. 34, the expression of CD3 mRNA in HO-1 treated animals was significantly augmented when compared to normal pregnant animals. It is tempting to speculate that this augmentation is due to a migration of lymphocytes into the feto-maternal interface. As we could observe higher Th2 than Th1 levels it is then postulated that more Th2 lymphocytes generated in the periphery (Fig. 33A and 34A) reach the feto-maternal interface (Fig. 33B).

Table 9 : similar levels of CD3 mRNA in placental tissues between all groups as measured by real time RT-PCR

	N.P (n=3)	*S.A. (n=5)*	*S.A. + AdHO1/GFP (n=5)*	S.A. + AdEGFP (n=4)
Mean	0.0003161	0.0002290	0.0003148	*0.001146*
SD	0.0005072	0.0002357	0.0003607	*0.001094*
Median	*0.0000277*	*0.0001757*	*0.0002286*	*0.0007203*

N.P.= Normal Pregnancy; S.A.= Spontaneous abortion. No significant differences were found between the groups as analyzed by the Kruskall-Wallis non-parametric test between all groups.

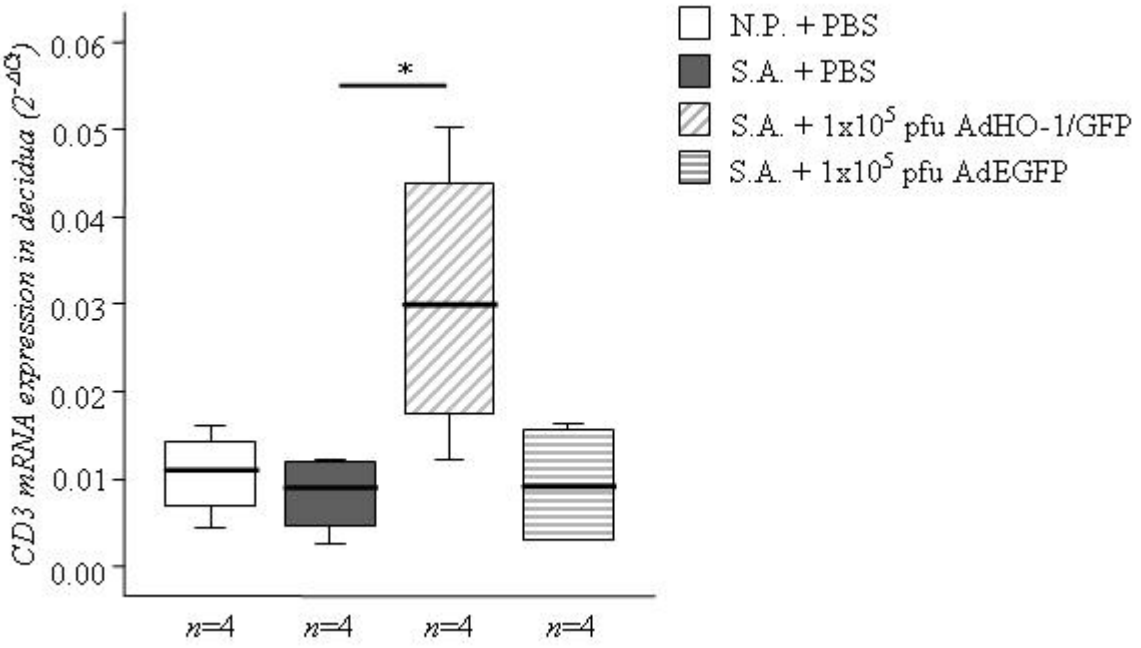


Fig. 34 : The systemic application of AdHO-1/GFP led to an augmentation of CD3 mRNA in the decidua as analyzed by real time PCR normalized to β-actin.

Decidual expression of CD3 mRNA as analyzed by real time PCR normalized to b-actin. Data are represented by medians ± 75% quartiles. N.P.= Normal Pregnancy; S.A.= Spontaneous abortion. Significant differences between groups are indicated as asterisks above lines. *p≤ 0.05, as analyzed by the Kruskall-Wallis non-parametric test between all groups, followed by the Mann-Whitney U-test for two particular groups.

CD3 expression at protein level was analyzed in placental tissue by immunohistochemistry. Unfortunately, $CD3^+$ cells were rare to found. Since in many samples no cells could be found, it was not feasible to quantify the number of $CD3^+$ cells. Nevertheless, samples of animals

receiving AdHO-1/GFP did not show massive infiltration of $CD3^+$ lymphocytes, supporting the RT-PCR data with no differences in CD3 expression in placental tissue between the groups.

4.2.6 Levels of FoxP3 and TGF-β mRNA as measured by real time RT-PCR

Many authors have proposed a link between HO-1 and regulatory T cells (Treg) (Pae *et al.*, 2003; Choi *et al.*, 2005), although this is still controversial. In order to clarify whether in our animal model HO-1 was able to up-regulate the number of Treg at the feto-maternal interface, the markers TGF-β and FoxP3 were analyzed by real time RT-PCR in placental tissue. FoxP3 is considered to be a specific marker that indicates the presence of Treg as it is constitutively expressed in Treg but not in effector T cells. TGF-β is normally secreted by certain types of Treg and augmented levels of any of these markers or of both markers together may indicate the presence of Treg. As shown in Table 10 no augmentation of TGF-β mRNA was observed when injecting AdHO-1/GFP, but there was an augmentation in the levels of TGF-β in the group receiving AdEGFP when compared with the group receiving AdHO-1/GFP. Although these results were not expected as it would mean that the sole injection of an adenovirus is protective, one have to keep in mind that TGF-β is mainly regulated post-transcriptionally, and mRNA levels may not reflect protein levels, hence may not count for the *in vivo* situation. Unfortunately, it was impossible to measure protein levels by IHC or Western Blot.

Table 10 : No differences in the mRNA levels of TGF-β in placental tissue between the groups as meaured by real time RT-PCR

		***N.P* (n=5)**	***S.A.* (n=6)**	***S.A.* + *AdHO1/GFP* (n=5)**	**S.A. + AdEFP (n=5)**
TGF-β	*Mean*	0.1278	0.1041	0.05937	*0.1624*
	SD	0.1106	0.06807	0.05241	*0.02397*
	Median	*0.1344*	*0.08931*	*0.04123*	*0.1560**

N.P.= Normal Pregnancy; S.A.= Spontaneous abortion. *p≤0.05 as analyzed by the Kruskall-Wallis non-parametric test between all groups, followed by the Mann-Whitney U-test for two particular groups. Differences were found between the group receiving AdEGFP and the group receiving AdHO-1/GFP.

Levels of FoxP3 mRNA were found to be comparable between all groups analyzed (Table 11). No relationship could be found between the application of the adenoviral vector containing HO-1 and Treg. However, more studies should be carried out before excluding a connection between these two systems in this particular model.

Table 11 : No differences in the mRNa levels of FoxP3 in decidual tissue between the groups

		N.P ***(n=3)*** [§]	***S.A.*** ***(n=2)*** [§]	***S.A. +*** ***AdHO-1/GFP*** ***(n=3)*** [§]	**S.A. + AdEGFP (n=3)** [§]
FoxP3	*Mean*	4.00×10^{-5}	1.24×10^{-4}	1.23×10^{-4}	9.59×10^{-5}
	SD	2.88×10^{-5}	1.91×10^{-5}	6.35×10^{-5}	4.29×10^{-5}
	Median	2.43×10^{-5}	1.24×10^{-4}	9.06×10^{-5}	9.61×10^{-5}

N.P.= Normal Pregnancy; S.A.= Spontaneous abortion. No significant differences were found between the groups as analyzed by the Kruskall-Wallis non-parametric test between all group. [§]Only samples with detectable levels of FoxP3 were included.

4.2.7 Measurement of apoptosis at the feto-maternal interface

HO-1 and its products are known to exert anti-apoptotic effects in many different models. In order to see if HO-1 also has an anti-apoptotic effect at the feto-maternal interface, apoptosis was measured by two different methods, namely caspase-3 activity in tissue homogenates and TUNEL staining in tissue slides. The measurement of Caspase-3 activity of whole placental homogenates showed a slight augmentation in the caspase-3 activity, thus in apoptosis, in placentas from abortion-prone animals when compared to placentas from normal pregnant mice. The caspase-3 activity could be diminished after injection of AdHO-1/GFP, suggesting an anti-apoptotic effect of the HO-1 therapy that may involve a caspase-3 dependent pathway. The levels of caspase-3 activity were also diminished when compared to the levels measured in mice receiving an AdEGFP control vector. Besides, the placental homogenates from animals receiving the AdEGFP control vector showed even a slight augmentation when compared to the abortion-prone group. Results for caspase-3 activity are shown in Fig. 35. The measurement of caspase-3 activity gives an idea of the rate of apoptosis in the tissue that occurred in a caspase-3 dependent way. However, other mechanisms of apoptosis may occur, and this was the reason why apoptosis was also measured by TUNEL technology.

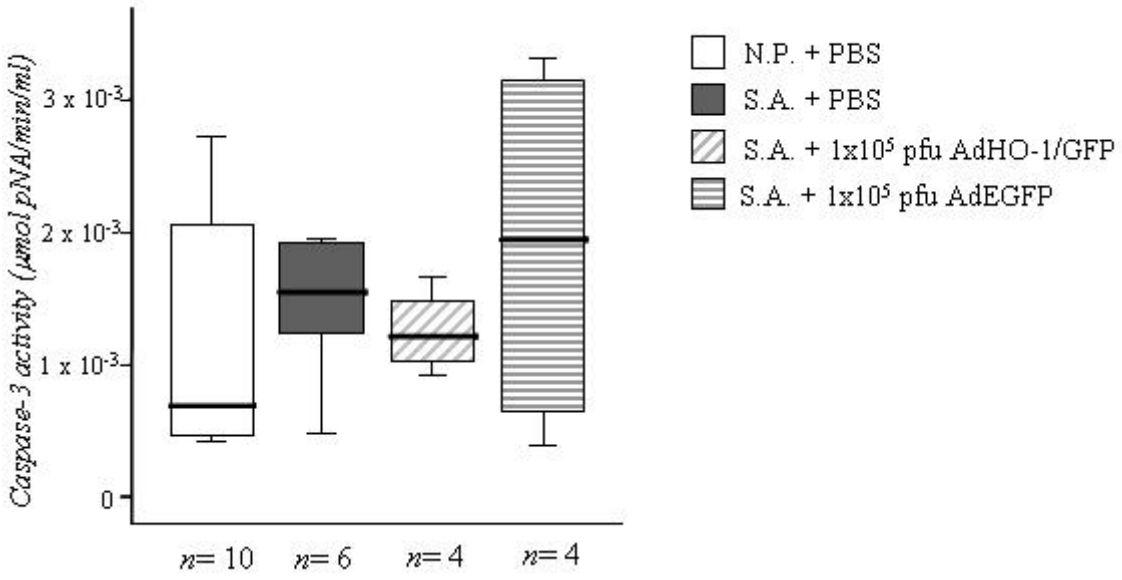


Fig. 35 : Diminished caspase-3 activity in placental tissue of animals receiving the AdHO-1/GFP vector

Data are represented by medians ± 75% quartiles. N.P.= Normal Pregnancy; S.A.= Spontaneous abortion; Circles and/or asterisks above box plots indicate outliers. No significant differences were found as analyzed by the Kruskall-Wallis non-parametric test between all groups.

By using the TUNEL technology, the number of apoptotic cells/mm^2 in the placenta could be determined by light microscopy based on the morphology of the stained nuclei of the cells, and results are depicted in Fig.36. Placentas from abortion-prone mice presented significant higher number of apoptotic cells when compared to placentas from normal pregnant animals.

The application of AdHO-1/GFP led to a significant diminution of the number of apoptotic cells when comparing to placentas from abortion-prone mice receiving PBS or AdEGFP, reinforcing the hypothesis of an anti-apoptotic effect of the HO-1 therapy. The fact that a big diminution in the number of apoptotic cells was found in the group receiving AdHO-1/GFP, whereas the difference obtained in the caspase-3 activity was only slight and not significant may suggest that the AdHO-1/GFP could have acted also in a caspase-3 independent pathway. A representative picture of the TUNEL staining in placental tissue is shown in Fig. 37A. Fig. 37B shows the negative control of the staining.

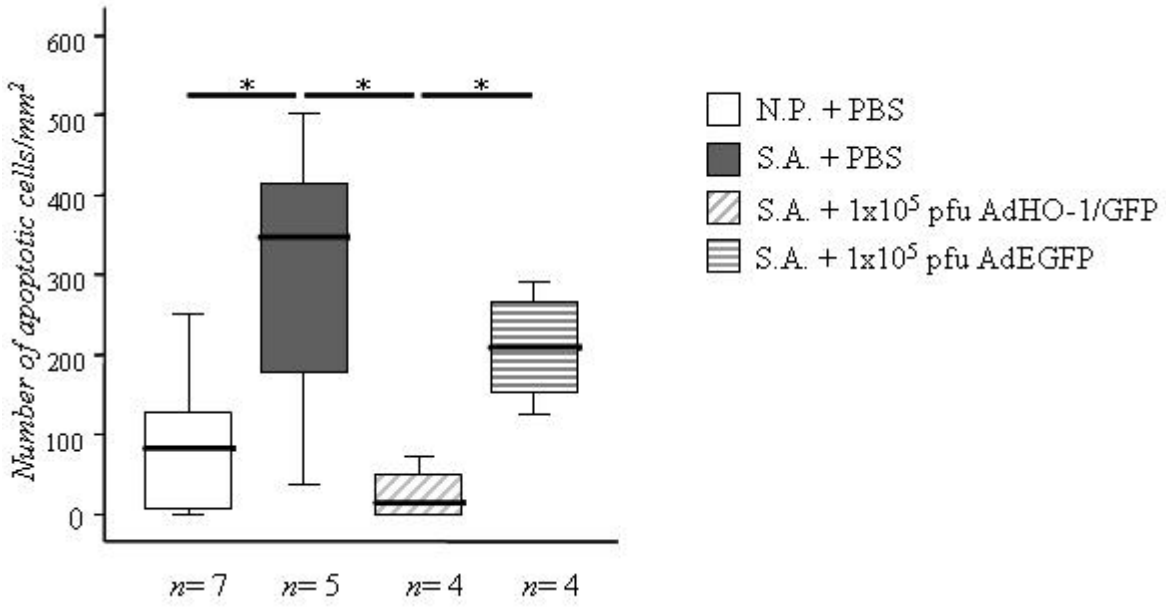


Fig. 36 : The up-regulation of HO-1 led to a significant diminution in the number of apoptotic cells in the placenta as measured by TUNEL

Number of apoptotic cells/mm^2. For each section, positive cells were counted in at least 20 fields using a 200x magnification (20x objective and 10x ocular). Data are represented by medians ± 75% quartiles. N.P.= Normal Pregnancy; S.A.= Spontaneous abortion. Significant differences between groups are indicated as asterisks above lines. *p≤ 0.05 as analyzed by the Kruskall-Wallis non-parametric test between all groups, followed by the Mann-Whitney U-test for two particular groups.

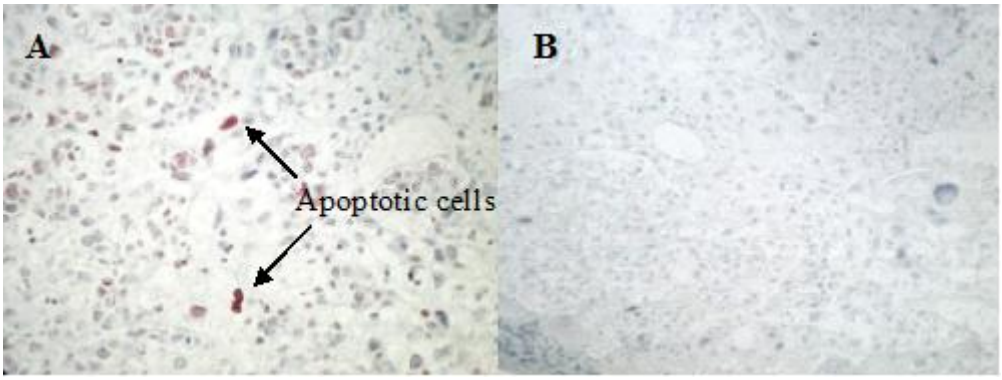


Fig. 37 : Representative example of the TUNEL staining in placental tissue

A) sample containing apoptotic cells;

B) negative control; pictures taken at 20X objective and 10X ocular amplification.

4.2.8 Measurement of mRNA of pro- and anti-apoptotic molecules by real time RT-PCR

Since mice receiving AdHO-1/GFP presented diminished apoptosis, it was interesting to go into a deeper analysis and try to elucidate by which mechanism this prevention of apoptosis occurred. For that, mRNA levels of Bag-1 and further anti-apoptotic molecules such as Bcl-xl and Bcl-2 in decidual and placental samples were analysed by real time RT-PCR. As depicted in Fig. 38, Bag-1 mRNA levels were slightly diminished in decidual tissues from the abortion-prone group when compared to the normal pregnancy group. Interestingly, AdHO-1/GFP treatment could revert the Bag-1 down-regulation ($p<0.05$), confirming an anti-apoptotic effect of the HO-1 therapy. Levels of mRNA for Bcl-xl and Bcl-2 molecules were not affected by the AdHO-1/GFP treatment and no differences between the groups were found (data not shown).

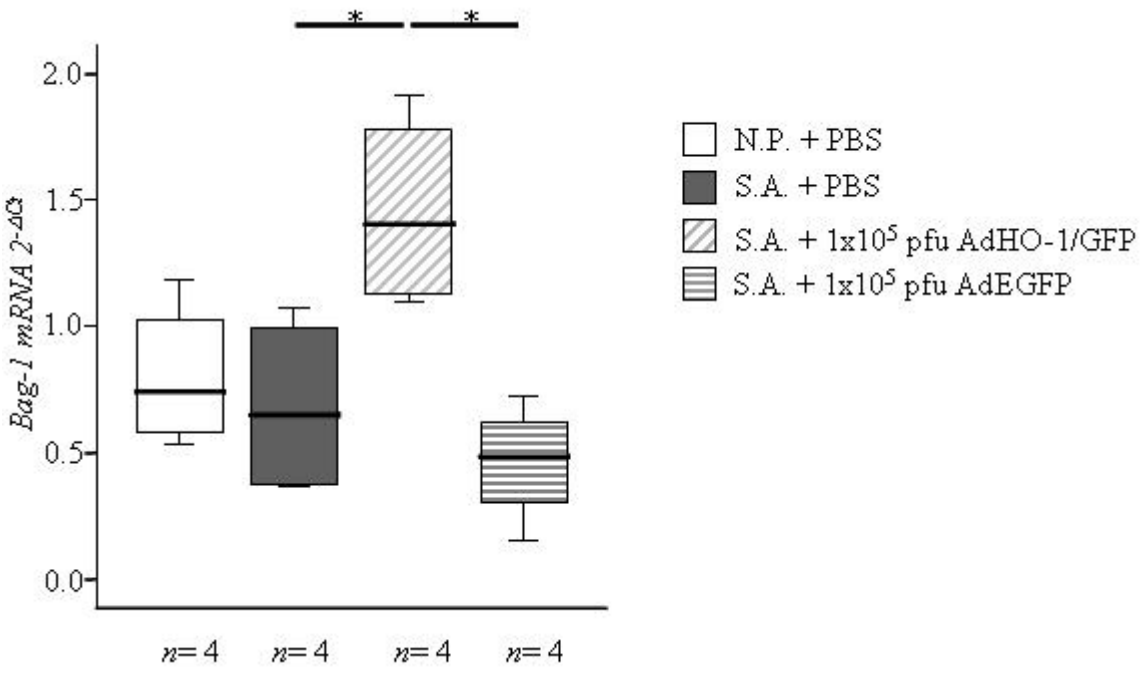


Fig. 38 : Up-regulation of HO-1 led to a significant augmentation of the mRNA levels of the anti-apoptotic molecule Bag-1 as measured by real time RT-PCR.

Data are represented by medians ± 75% quartiles. N.P.= Normal Pregnancy; S.A.= Spontaneous abortion. Significant differences between groups are indicated as asterisks above lines. *$p \leq 0.05$ as analyzed by the Kruskall-Wallis non-parametric test between all groups, followed by the Mann-Whitney U-test for two particular groups.

4.2.9 Vascular endothelial growth factor (VEGF)

The HO-1 molecule is known to be related to angiogenesis. In this regard, it has been shown by others (reviewed in Dulak *et al.*, 2004) that an up-regulation of HO-1 leads to an augmentation of the angiogenic factor VEGF and viceversa (Bussolati *et al.*, 2004). In order to analyze whether the beneficial effect obtained by the up-regulation of HO-1 was related to angiogenesis, the expression of VEGF was measured at a single cell level in placental tissue by immunhistochemistry. The results obtained with this method are shown in Fig. 39, where it can be seen that animals receiving low doses of AdHO-1/GFP showed a slight augmentation in the number of cells expressing VEGF, when compared to all other groups, suggesting that HO-1 may have also angiogenic effects in this particular model.

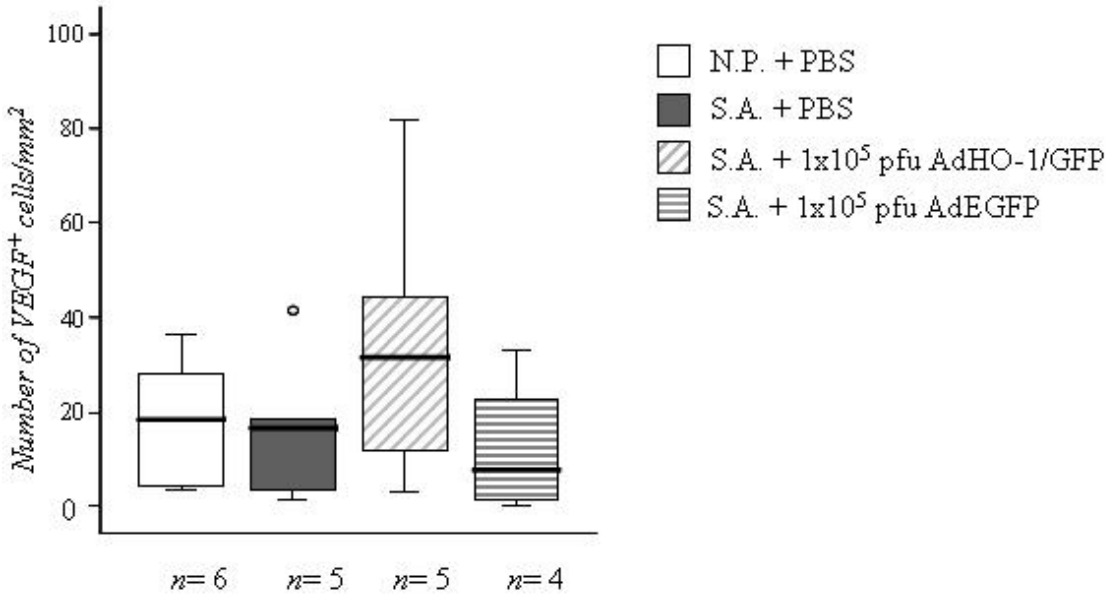


Fig. 39 : Slight augmentation in the number of VEGF+ cells in placentas of animales treated with AdHO-1/GFP as analyzed at single cell level by IHC.

Number of $VEGF^+$ cells/mm^2. For each section, positive cells were counted in at least 20 fields using a 200x magnification (20x objective and 10x ocular). Data are represented by medians ± 75% quartiles. N.P.= Normal Pregnancy; S.A.= Spontaneous abortion. No significant differences were found between groups as analyzed by the Kruskall-Wallis non-parametric test between all groups.

An example of the immunohistochemical staining for VEGF is shown in Fig. 40, where a sample containing $VEGF^+$ cells (A) is depicted next to the negative control of the staining (B).

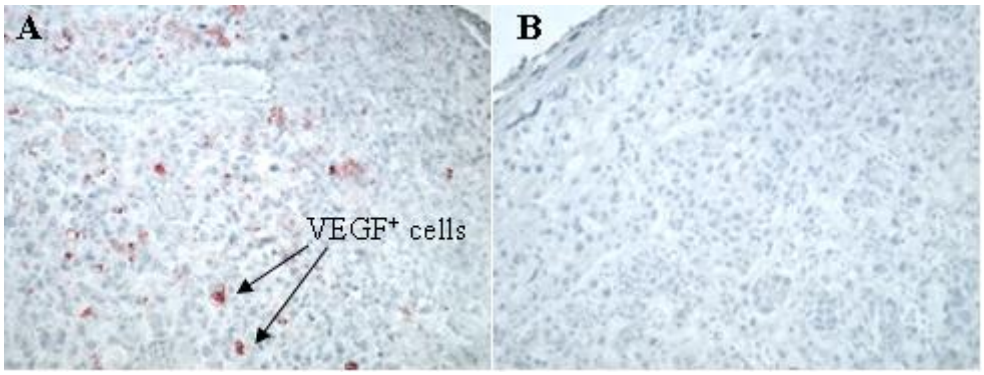


Fig. 40 : Representative example of the VEGF expression in placental tissue as measured by immunohistochemistry in paraffin-embedded tissue.

A) sample containing VEGF positive cells; B) negative control of the staining. Pictures taken at 20X objective and 10X ocular amplification.

4.3 *In vitro* studies using the Rcho-1 trophoblast cell line

4.3.1 Effect of CoPPIX and ZnPPIX on the viability of trophoblast precursor cells

The Rcho-1 cell line is a trophoblastic stem cell line that differentiates into giant cell line upon different culture conditions. This cell line is useful to study whether HO-1 is necessary for these cells to survive and differentiate into giant cells in the context of this thesis. For this purpose, the effect of CoPPIX and ZnPPIX was analyzed in the culture of undifferentiated Rcho-1 cells. ZnPPIX is known to down-regulate HO-1 *in vivo* as well as *in vitro*. CoPPIX is known to up-regulate HO-1 in vivo, whereas *in vitro* is controversial if this up-regulation takes place (reviewed in Ryter *et al.*, 2006). Since no reports of the effect of CoPPIX or ZnPPIX on trophoblast cells were found in the literature, different conditions were tested in order to use the optimal concentration of ZnPPIX and CoPPIX for these cells. The results on HO-1 expression using the different concentrations and incubation time are shown in Fig. 41.

As it can be observed in the western blot example (Fig 41), Rcho-1 cells express very high basal levels of HO-1. A down-regulation of HO-1 can be observed already with 50 mM ZnPPIX at 24h. Unlike ZnPPIX, CoPPIX had no effect on HO-1 levels in these cells as HO-1 expression remained at basal levels. Cells treated with CoPPIX were used then as a control for the assay, in order to analyze whether the effects observed with ZnPPIX are due to the down-regulation of HO-1 or to a putative toxic effect of the porphyrin.

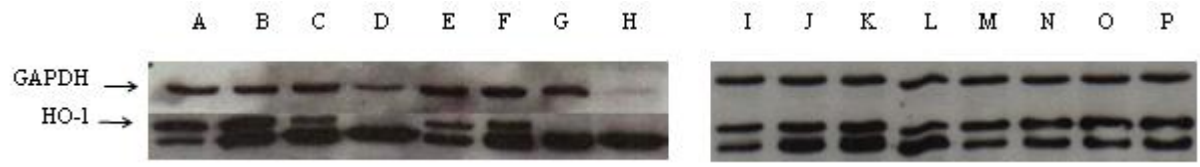


Fig. 41 : Example of the Western Blot analysis of Rcho-1 cells treated with CoPPIX or ZnPPIX

A to D: 12 h after ZnPPIX treatment (0, 25, 50 and 100 µM respectively).

E to H: 24 h after ZnPPIX treatment (0, 25, 50 and 100 µM respectively)

I to L: 12 h after CoPPIX treatment (0, 25, 50 and 100 µM respectively).

M to P: 24 h after CoPPIX treatment (0, 25, 50 and 100 µM respectively).

It is unknown the reason why the antibody against HO-1 gave two bands in these samples. Nevertheless, the upper band (corresponding to 32 kDa) was taken into consideration.

In a first assay, cell viability of the trophoblast stem cell was analyzed after 48 h of treatment with medium, ZnPPIX or CoPPIX by tryphan blue exclusion.

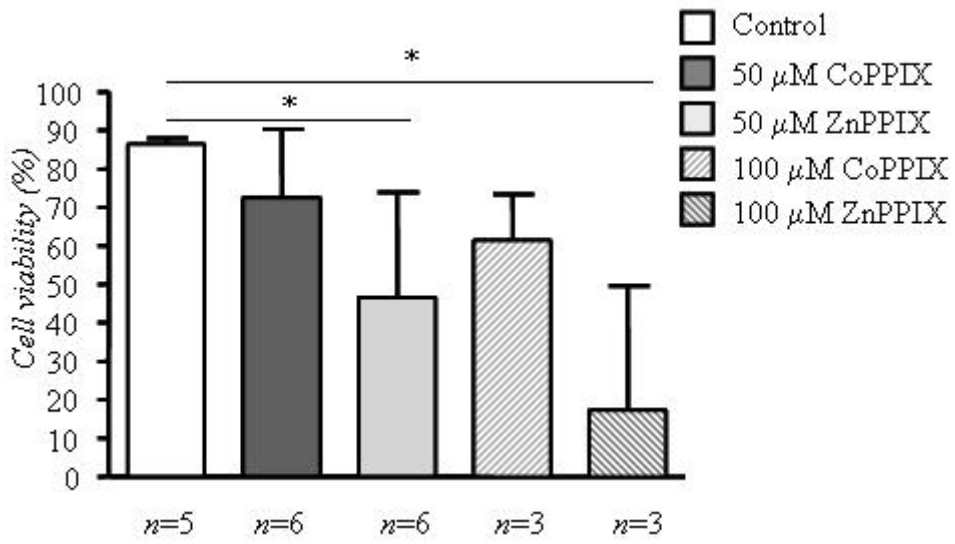


Fig. 42 : HO-1 down-regulation leads to diminished viability of the trophoblast stem cells as measured by trypan blue exclusion.

Data are represented by median & range. Significant differences between groups are indicated as asterisks above lines. *$p \leq 0.05$ as analyzed by the Kruskall-Wallis non-parametric test between all groups, followed by the Dunn´s Multiple Comparison Test for two particular groups.

As it can be observed in Fig. 42, a significant diminution in the viability of the cells was obtained 48 h after cells were treated with 50 µM or 100 µM ZnPPIX. This effect cannot be due

to the toxic effect of the porphyrin, since cells treated with CoPPIX did not show a significant diminution in the cell viability. Since ZnPPIX is known to block the expression of HO-1, it can be assumed that HO-1 is necessary for the survival of trophoblast precursor cells.

4.3.2 Effect of CoPPIX and ZnPPIX on the differentiation of trophoblast precursor cells into giant cells

The effect of CoPPIX and ZnPPIX in the differentiation of Rcho-1 cells into giant cells was analysed applying CoPPIX or ZNPPIX during the differentiation process. Differentiated cells show a characteristic morphology, and they can be well recognized from undifferentiated cells. Differentiated cells (giant cells) are considerably bigger that undifferentiated cells, and they are normally multinucleated or bear a big nucleus. Differentiation of these cells is normally obtained after 1 week of culture in medium containing 10% horse serum.

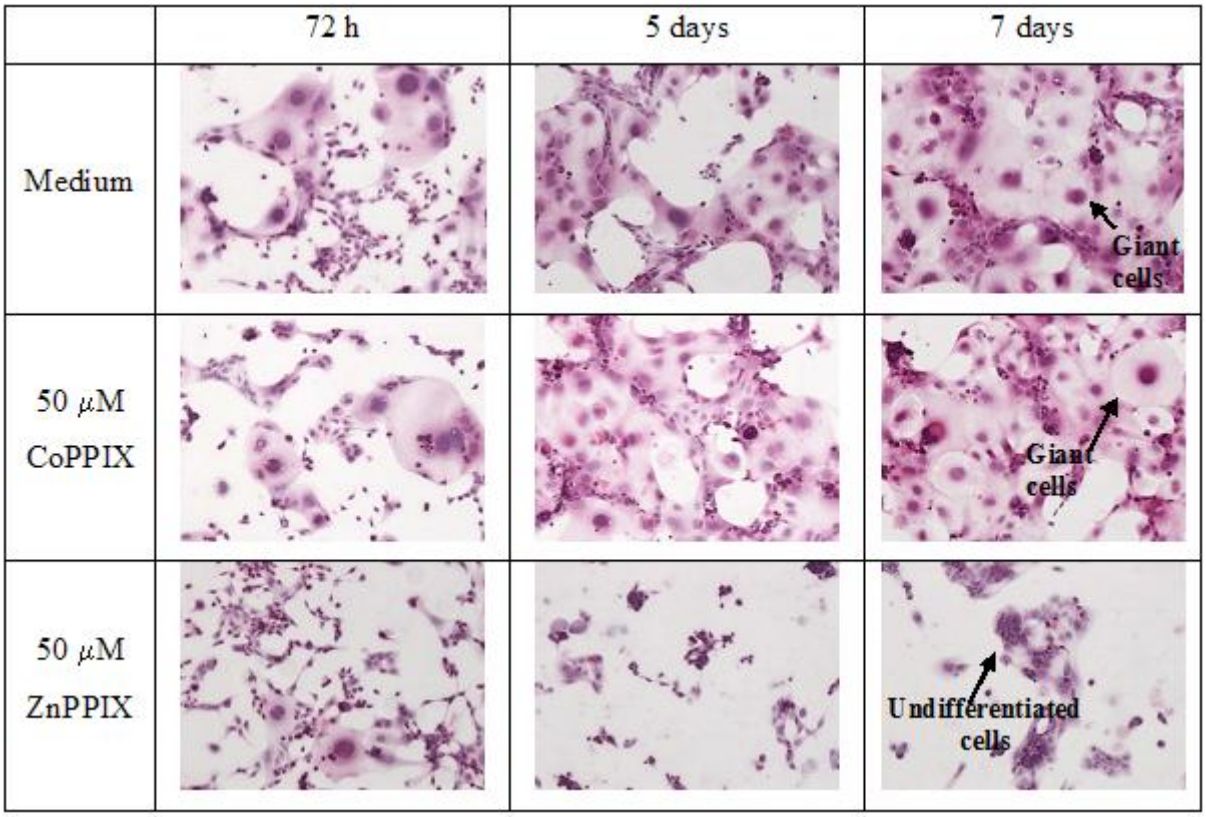


Fig. 43 : Stem cells treated with ZnPPIX were unable to differentiate into giant trophoblast cells as analyzed by light microscopy.

Pictures taken after 72 h, 5 days and 7 days of culture under differentiation media. Cells treated with normal media as well as cells treated with CoPPIX are able to differentiate into giant cells, whereas cells treated with ZnPPIX are unable to differentiate into a mature state.

As observed in Fig. 43, the morphology of the cells start changing already at 72 h of culture and the differentiation is complete at 7 days of culture with medium including horse serum. When applying CoPPIX (Fig. 43), no significant changes in the morphology of the cells are observed when compared to cells treated with only differentiation medium. However, when applying ZnPPIX (Fig 43), which blocked HO-1, the cells are unable to differentiate into giant cells, pointing out that HO-1 is indispensable for trophoblast precursor cells to differentiate into a mature phenotype. This effect could be due to the toxic effects of ZnPPIX, but this is not rather the case, since cells receiving CoPPIX, where the same toxic effect is applied, did not show alterations in their differentiation capacity.

4.4 *In vitro* studies using mouse T cells over-expressing HO-1

4.4.1 Generation of the packaging cell lines

With the aim to transduce lymphocytes with HO-1 for testing their in vitro or in vivo ability to suppress maternal T cell responses, we proceed to first generate packaging cell lines expressing HO-1. Since retroviral vectors are suited for transduction of T cells, two different packaging cell lines overexpressing the HO-1 transgene were obtained. These cell lines showed the following viral titers:

GP+E HO-1: 6.10^6 cfu/ml.

GP+E HO-1iresEGFP: 4.10^6 cfu/ml.

After limiting dilution, 14 clones of the packaging cell line HO-1 and 9 clones of the HO-1iresEGFP packaging cell line were selected. The selection criterion was based on the Western Blot intensity of HO-1 observed for each sample. Representative examples of different HO-1 protein expression patterns are shown in Fig.44. In A) it can be observed the difference in the HO-1 expression between the packaging cell line without the HO-1 transgene, and both packaging cell lines obtained. These packaging cell lines were further cloned, and examples of the different HO-1 expression in the clones are illustrated in B).

After selecting the highest producer clones, the viral titers were determined as already described and the results are summarized in the following table (Table 12).

Since there is not always a correlation between the transgene expression and the viral titer, it was necessary to select those clones that show not only a high expression of the transgene but also have a high viral titer.

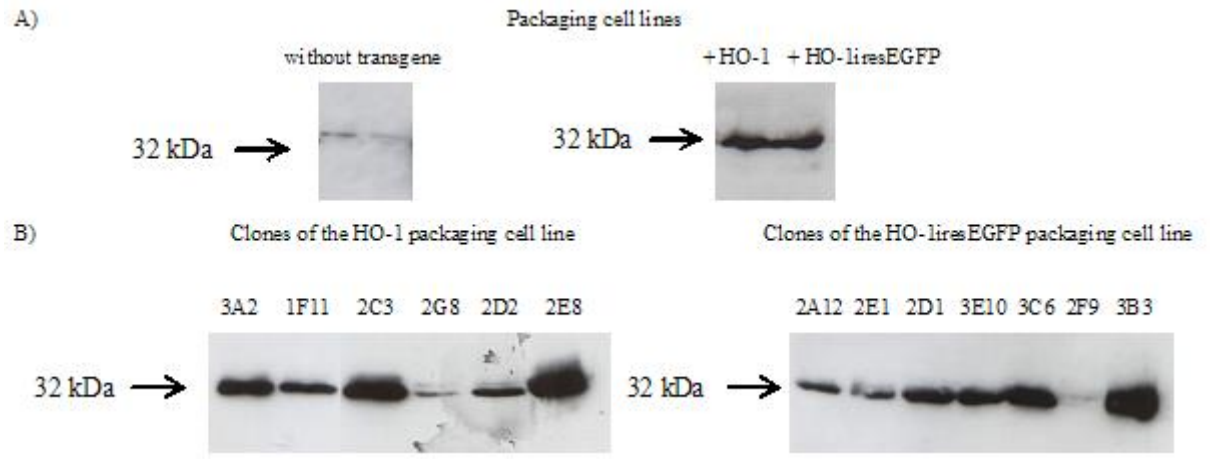


Fig. 44 : Cells from the packaging cell line were efficiently transfected to produce high amounts of HO-1 as observed by Western Blot analysis.

A) HO-1 expression in the packaging cell lines prior and after transfection. B) HO-1 expression in the different clones from the HO-1 and the HO-1iresEGFP packaging cell lines.

Table 12 : Viral titers of the different clones of the HO-1 over-expressing packaging cell lines

A

Clone	*Viral titer (cfu/ml)*
1D10	3.10^7
1E12	3.10^6
1H12	3.10^5
2A11	2.10^7
2C11	3.10^5
2C3	7.10^6
2D12	1.10^6
2E8	9.10^6
3A2	1.10^7
3B10	1.10^6
3B6	2.10^7
3B7	4.10^7
3D8	1.10^7
3H1	1.10^7

B

Clone	*Viral titer (cfu/ml)*
1H7	4.10^5
2A10	2.10^7
2D1	6.10^6
2D7	1.10^8
2G2	1.10^6
3B3	1.10^8
3E1	1.10^7
3E10	8.10^7
3G8	8.10^7

A) Clones from the HO-1 packaging cell line; B) Clones from the HO-1iresEGFP packaging cell line

After careful analysis of the results from Western Blots and viral titers determination, the clone 3B3 was selected as an appropriate one to be used in the transduction of the T-lymphocytes with the HO-1iresEGFP, since it shows a high expression of the protein, and an excellent viral titer. In addition, it harbours the advantage of the EGFP as a reporter gene to control the transduction efficiency. From the HO-1 packaging cell line, the clone 3D8 was selected.

4.4.2 Transduction of mouse T cells

Many conditions for mouse T cell transduction with the retroviral particles were tested. The highest transduction efficiency (**7.91%**) was obtained when CD4+ T cells were transduced with 500 ml of virus concentrate, in a final volume of 1.5 ml in a 12-well plate. The virus concentrate was obtained by means of centrifugation in a Vivaspin column. An example of the efficiency of virus transduction, as measured by flow cytometry, is shown in Fig. 45. All other conditions tested (explained in materials and methods) were unsuccessful or led to even lower transduction efficiency (around 2%).

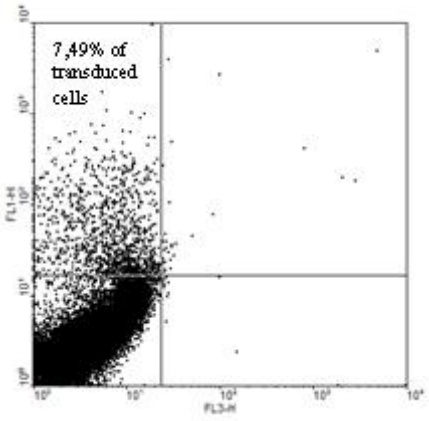


Fig. 45 : The highest transduction efficiency was obtained by using a virus concentrate as analyzed by flow cytometry

FL-1H measures the cells that are positive for HO-1iresEGFP (transduced cells). In this example, 7.49% of the total cells were efficiently transduced.

These transduced cells could theoretically be selected by G-418. Although the selection seemed to work, the number of cells remaining alive was to low to be maintained in culture, and the cells died after few days. This was repeatedly observed in several approaches. Since the G-418 selection was not feasible, the obtainment of a sufficient amount of transduced cells

would have required the sacrifice of more than one animal in order to obtain a sufficient initial amount of cells. Additionally, an excessive production of virus supernatant would have been necessary in order to obtain enough amount of virus concentrate. All these inconvenient led us to conclude that the transduction of mouse T cells with a retroviral vector was not a feasible for our proposed experimental settings, at least using this retroviral vector. We decided then to try alternative methods, like protein transfection, in order to obtain T cells over-expressing HO-1.

4.4.3 Protein Transfection

Protein transfection is still not described in the literature for mouse primary T cells as it is for other cells like murine and human fibroblasts. In order to establish the better condition for transfection, a positive control using β-galactosidase was used (suggested by the manufacturer of the protein transfection reagent). As mentioned in Materials and Methods, different cell number, protein content and incubation times were analyzed. Fig.46 depicts the different results obtained.

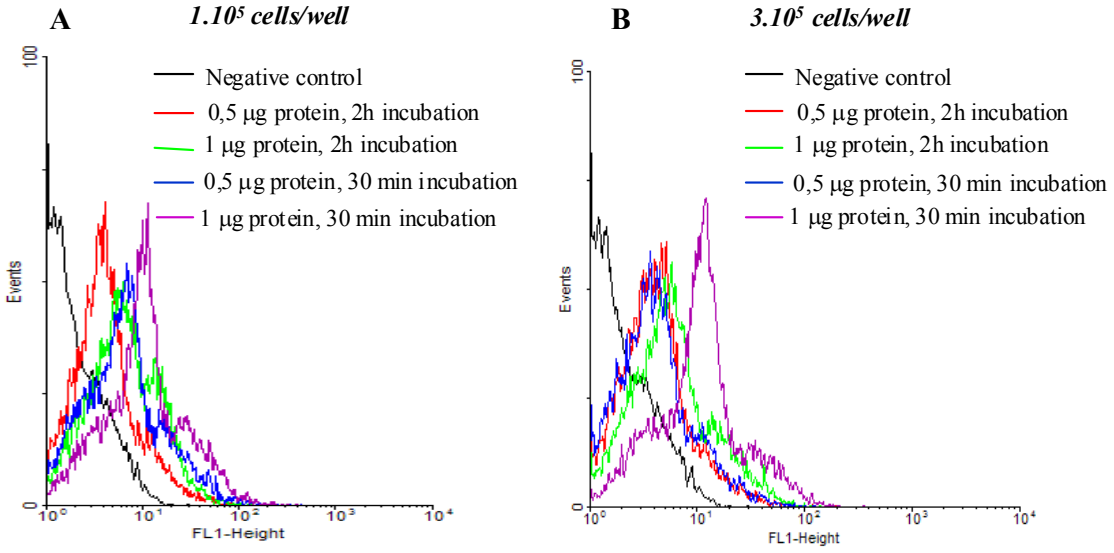


Fig. 46 : Up to 80% of the cells were efficiently transfected with the b-gal protein using the protein transfection technology as analyzed by flow cytometry.

Data are shown by histogram. FL1-Height measures the cells that show b-galactosidase activity. Fig. A shows the different conditions tested for $1x10^5$cells/well, whereas Fig. B shows the conditions tested for $3x10^5$cells/well. Up to 80.95% of the cells (purple line, diagram B) were efficiently transfected.

As mentioned in Materials and Methods, the condition mentioned in 2.2.2.10 was selected as optimal for protein transfection. Transfection with HO-1 protein could unfortunately not be measured by flow cytometry, and the optimal protein amount was determined by western blot. As can be seen in Fig. 47, transfected cells showed higher expression of HO-1 protein when compared to untransfected cells, and the amount of 2 μg of protein was selected as optimal for the further experiments.

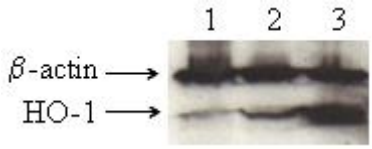


Fig. 47 : HO-1 protein was efficiently transfected into $CD4^+$ cells as determined by Western Blot analysis

1) Untransfected $CD4^+$ T cells

2) $CD4^+$ T cells transfected with 1 μg of HO-1 protein

3) $CD4^+$ T cells transfected with 2 μg of HO-1 protein

4.4.4 *In vitro* analysis of the effect of HO-1^+ T cells on the proliferation of T cells

The idea to use cells over-expressing a given molecule as therapy for transplantation (Hammer *et al.*, 2000) gave us the idea of using $CD4^+$ T cells in an abortion-prone setting. For that reason, the effect of $CD4^+$ T cells over-expressing HO-1 on cells from an abortion-prone female was analyzed. Since HO-1 has been described as having anti-proliferative effects, the proliferation of these cells (called from now on "control cells") was analyzed by flow cytometry using the CFSE membrane dye. For doing this, cells from a CBA/J female previously mated with a DBA/2J male were isolated from lymph nodes, spleen, and thymus and stained with CFSE. From the thymus, $CD4^+CD25^+$ and $CD4^+CD25^-$ were isolated and separately tested. As previously mentioned, the female was pregnant on day 5 from a DBA/2J male, meaning that cells from this female have already encountered male antigens and are supposed to generate an antigen specific reaction. In order to stimulate the culture and to simulate the continuous encounter with male antigens, DBA/2J cells previously treated with mitomycin C were used as stimulators.

As depicted in Fig 48, and contrary to our expectations, no differences in the proliferation of control cells could be found when $CD4^+$ T cells over-expressing HO-1 were put in contact with cells from lymph nodes, spleen or thymus of a CBA/J pregnant female. A significant difference was observed in the proliferation of spleen cells, but this diminution was also observed when T_{MOCK} cells were used. The presence of these cells (both $T_{HO\text{-}1+}$ or T_{MOCK} cells) did also not influence the expression of different markers as CD69 or CD25 (data not shown).

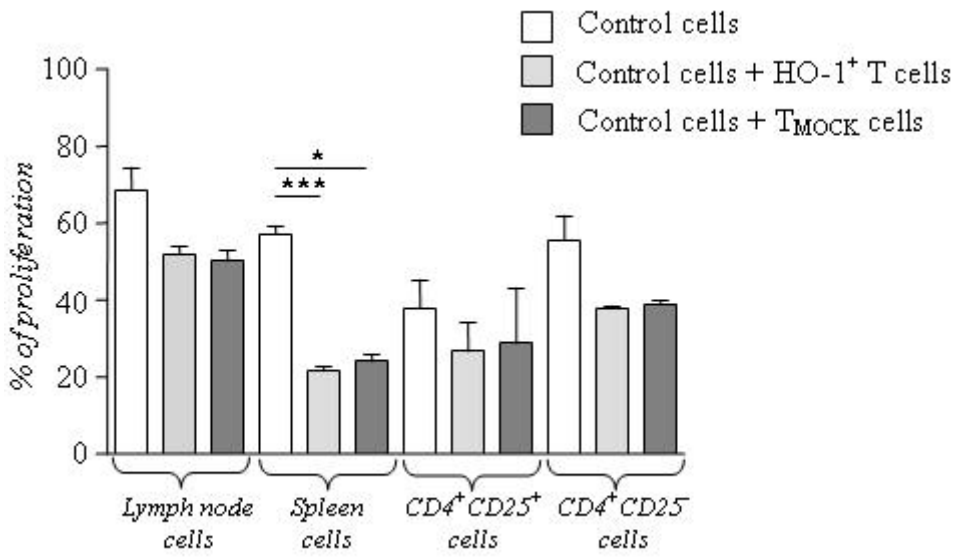


Fig. 48 : No effect of $CD4^+$ T cells overexpressing HO-1 on the proliferation of control T cells as measured in a MLC.

Data are represented by mean ± SD. Significant differences between groups are indicated as asterisks above lines. *$p \leq 0.05$ and ***$p \leq 0.001$ as analyzed by the Kruskall-Wallis non-parametric test between all groups, followed by the Dunn´s Multiple Comparison Test for two particular groups.

The effect of these cells was also tested *in vivo*. For this purpose, DBA/2J-mated CBA/J females were intravenously injected with $CD4^+$ T cells over-expressing HO-1 or with T_{MOCK} cells in day 5 of pregnancy. Also *in vivo*, no effect of these cells could be observed, since the abortion rates remained similar between the groups.

From this part of the study, it can be concluded that in this particular model, a cell therapy with lymphocytes over-expressing HO-1 was not adequate for the purpose of reducing the abortion rate. However, one has to keep in mind that even when HO-1 was expressed in the T cells, the transfected protein may not have been active. Unfortunately we were not able to

measure HO-1 activity in these cells to confirm our hypothesis. Other possibility is that T cells are not the right target for over-expressing HO-1. Dendritic cells would be a rather better choice since it is known that HO-1 is expressed in them and that an up-regulation of HO-1 in these cells is capable of maintaining them in an immature state (Chauveau *et al*., 2005).

4.5 Studies using heme oxygenase-1 deficient mice

4.5.1 Analysis of the progeny of different mating combinations of mice partially or totally deficient on heme oxygenase-1

The use of transgenic animals lacking a given molecule represents a useful tool to analyze the effect that this produces in the organism. In pregnancy, it was essential for the purpose of this thesis to analyze the effect of the lack of HO-1 on different stages of pregnancy.

As already mentioned in the literature, breeding between homozygous *Hmox1* deficient mice does not yield progeny (Poss and Tonegawa, 1997), and mice genetically deficient in Heme Oxygenase-1 (*Hmox1* deficient mice, or *Hmox1*$^{-/-}$ mice) are partially embryonic lethal (Yet *et al*., 1999), suggesting a key role of HO-1 in the onset of pregnancy. Additionally, when heterozygous mice (*Hmox1*$^{+/-}$) are mated for maintaining the colony, very low percentage of knockout animals (*Hmox1*$^{-/-}$) are usually born, usually between 5 and 10%, according to the literature and to personal communication of Prof. Soares. This unexpected variation from the 25% expected Mendelian rate may be due to different reasons:

1) The *Hmox1*$^{-/-}$ fetuses are rejected
2) The *Hmox1*$^{-/-}$ blastocyst present implantation impairments
3) Oocytes lacking *Hmox1* are not as easily fertilized as oocytes expressing *Hmox1*.
4) Sperm lacking *Hmox1* does not fertilize as easily as sperm expressing *Hmox1*.

For the purpose of analyzing if the *Hmox1*$^{-/-}$ fetuses are rejected and to know if there is a difference in the number of implantations or in the abortion rates regarding the different expression of *Hmox1*, the outcome of different combinations of *Hmox1*$^{-/-}$, *Hmox1*$^{+/-}$ and *Hmox1*$^{+/+}$ mice was analyzed. This was tested in a syngeneic as well as in an allogeneic context, whereas the female was from a different genotype, namely C57/BL6. Unfortunately, for this

combination it was only possible to use wild type females (*Hmox1*$^{+/+}$) as no knockout in C57/BL6 background were available at the moment of performing the experiments. The results obtained are shown in Table 13.

It has been described previously (Poss and Tonegawa, 1997; Yet *et al*., 1999) that no progeny can be achieved when mating *Hmox1*$^{-/-}$ mice. However, these studies did not analyze whether this phenomenon is due to no implantation or due to complete rejection of the implanted embryos. Due to the difficulties in the obtainment of *Hmox1*$^{-/-}$ animals, we did not perform groups using *Hmox1*$^{-/-}$ females.

Table 13 : *Hmox1* partial or total deficiency in the female or the male resulted in implantation failure or higher abortion rates.

Mating genotype (female x male)	Number of concepti (number of animals)	Abortion rate (median) only in those animals with visible implantations on day 12-14	Mean of Implantation sites (plugged females)	Number of animals with visible cocepti (plugged female)
Syngeneic (BALB/c x BALB/c)				
***Hmox1*$^{+/-}$ x *Hmox1*$^{-/-}$**	44 (11)	38.7 %	4 (11)	4 (11), 36.4 %
***Hmox1*$^{+/-}$ x *Hmox1*$^{+/-}$**	82 (7)	22.2 %	11.7 (7)	7 (7), 100 %
***Hmox1*$^{+/-}$ x *Hmox1*$^{+/+}$**	46 (6)	18.4 %	7.7 (6)	4 (6), 66.7 %
***Hmox1*$^{+/+}$ x *Hmox1*$^{-/-}$**	32 (5)	9.4 %	6.4 (5)	3 (5), 60 %
***Hmox1*$^{+/+}$ x *Hmox1*$^{+/-}$**	42 (5)	11.1 %	8.4 (5)	5 (5), 100 %
***Hmox1*$^{+/+}$ x *Hmox1*$^{+/+}$**	43 (8)	0 %	5.45 (8)	5 (8), 62.5%
Allogeneic (C57/BL6 x BALB/c)				
***Hmox1*$^{+/+}$ x *Hmox1*$^{-/-}$**	58 (8)	8,6 %	7.25 (8)	7 (8), 87.5%
***Hmox1*$^{+/+}$ x *Hmox1*$^{+/+}$**	43 (8)	0 %	5.40 (8)	5 (8), 62.5%

Abortion rates and implantation sites resulting from the mating of mice with different genotypes for Hmox-1. In the second row, the total number of concepti is depicted, with the total number of females shown between brackets. In the third and fourth row, the number of plugged females is indicated between brackets.

When females partially deficient in Hmox-1 (*Hmox1*$^{+/-}$) are mated with *Hmox1*$^{-/-}$ males, 4 out of 11 females that showed insemination plug were indeed pregnant, and showed an abortion rate of 38.7%. The fact that only 4 out of 11 females were pregnant suggests that *Hmox1*$^{-/-}$ male may present fertilization problems. Similarly, when *Hmox1*$^{+/+}$ females were mated with

Hmox1$^{-/-}$, 3 out of 5 females mated were pregnant, suggesting here that *Hmox1*$^{-/-}$ males may have fertilization problems. However, *Hmox1*$^{+/+}$ males also showed fertilization problems when fertilizing *Hmox1*$^{+/-}$ or *Hmox1*$^{+/+}$ females, since not all females showing insemination plug were pregnant. This suggests that these fertilization problems may not be exclusively associated with the lack of Hmox1 in the male.

When analyzing the abortion rates from the heterozygous females, an interesting result is observed, namely that the abortion rate is higher when less HO-1 is present in the mating combinations. When these females were mated with *Hmox1*$^{+/+}$ males, they presented an abortion rate of 18.4 %, with *Hmox1*$^{+/-}$ males the abortion rate was of 22.4 %, whereas with *Hmox1*$^{-/-}$ males the abortion rate was of 38.7 %. Although these differences are not significant, a tendency towards an augmentation on the abortion rate with the diminution of HO-1 in the system can be appreciated.

When *Hmox1*$^{+/+}$ females were mated with all three types of males, they showed very similar abortion rates in all three types of combinations, and the abortion rate was normal for syngeneic combinations (between 0 and 10% of abortion). This data strongly suggests that HO-1 is very important in the maternal site for a successful pregnancy, whereas its absence in the paternal site may not be as important as like in the maternal site.

When doing an allogeneic combination using *Hmox1*$^{+/+}$ C57/BL6 females mated either with *Hmox1*$^{-/-}$ or *Hmox1*$^{+/+}$ BALB/c males, most of the females presenting insemination plug were pregnant. When the male was deficient in *Hmox1*, an 8.6% of abortion was observed, against 0% of abortion obtained with the *Hmox1*$^{+/+}$ males, confirming again that the absence of HO-1 on the paternal side may not be as important as it is in the maternal site.

Analysis of the genotype of the fetuses and resorptions were performed in the groups of heterozygous females mated either with knockout or heterozygous males. Some of the fetuses from the other groups were randomly analysed and confirmed to be heterozygous, as expected. An example of the genotyping analysis performed by PCR is shown in Fig. 49.

The results of the analysis are shown in Fig. 50 for the *Hmox1*$^{+/-}$ x *Hmox1*$^{-/-}$ combination, and in Fig. 51 for the *Hmox1*$^{+/-}$ x *Hmox1*$^{+/-}$ mating combination.

Surprisingly, the mating of *Hmox1*$^{+/-}$ females with *Hmox1*$^{-/-}$ males lead only to heterozygous offspring. These results may suggest that *Hmox1*$^{-/-}$ blastocysts were unable to implant, since fetuses as well as resorptions were heterozygous. The fact that in this group only 4 out of 11

females showing insemination plug were pregnant suggests that *Hmox1*$^{-/-}$ sperm may present fertilization problems.

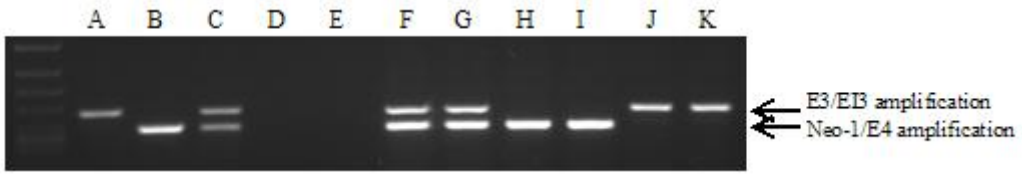


Fig. 49 : Example of amplification bands obtained in the genotyping of fetuses as analyzed in an Ethidium Bromide-stained gel after electrophoresis.

Amplification products of the PCR reactions for the E3/EI3 fragment (present in the wild type gene) and the Neo-1/E4 fragment (present in the modified gene) as analyzed in a 1% agarose gel containing ethidium bromide under ultraviolet transillumination.

A) Wild type control
B) Hmox1$^{-/-}$ control
C) Heterozygous control
D), E) Non Template Control (duplicate)
F), G) Heterozygous fetus (duplicate)
H), I) Hmox1$^{-/-}$ fetus (duplicate)
J), K) Wild type fetus (duplicate)

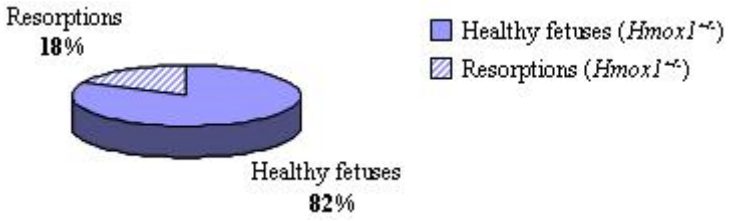


Fig. 50 : all fetuses and resorptions from the *Hmox1*$^{+/-}$ x *Hmox1*$^{-/-}$ combinations were heterozygous.

In the Hmox1$^{+/-}$ x Hmox1$^{-/-}$ combination, fetuses and resorptions from 2 pregnant females were genotyped.

On the other side, the mating of *Hmox1*$^{+/-}$ females with *Hmox1*$^{+/-}$ males led to the typical percentage of knockouts obtained from these matings, and this percentage is lower as the expected Mendelian rate of 25%. According to personal communication from Prof. Soares and to personal observation from the maintenance of the colony, this percentage is always between 3 and 10%. This reinforces the idea of inability of knockout embryos to implant, or

inability of females lacking partially HO-1 of providing essential conditions for fetuses to implant.

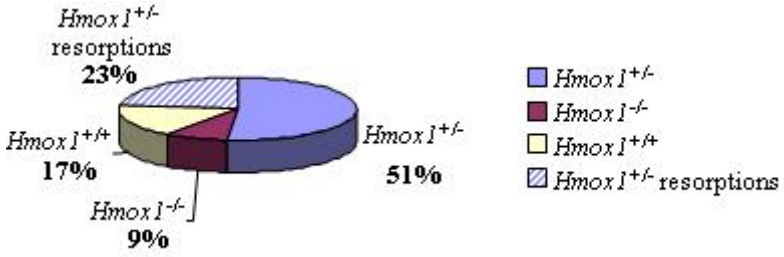


Fig. 51 : All resorptions from the *Hmox1*$^{+/-}$ x *Hmox1*$^{+/-}$ combination were heterozygous

In the Hmox1$^{+/-}$ x Hmox1$^{+/-}$ combination, fetuses and resorptions from 3 females were genotyped.

Interestingly, the resorptions were again heterozygous (Fig 51) suggesting that the lower percentage of *Hmox1*$^{-/-}$ fetuses obtained from the *Hmox1*$^{+/-}$ x *Hmox1*$^{+/-}$ mating is due to the lack of implantation of the *Hmox1*$^{-/-}$ blastocysts rather than to rejection of these fetuses.

4.5.2 Assessment of fertility of the *Hmox1*$^{-/-}$ females

4.5.2.1 Number of oocytes after hormonal stimulation

In the former experiment it could be observed that HO-1 is very important especially in the mother, for successful pregnancy to occur. This does still not explain why the mating of heterozygous mice does not give the expected Mendelian rate, since the fetuses that were rejected were heterozygous and not homozygous as expected, and does also not explain why homozygous mice do not give viable progeny. In order to assess if this can be due to sterility problems of the homozygous mice or to a defect or delayed ovulation, *Hmox1*$^{-/-}$ and *Hmox1*$^{+/+}$ females were first stimulated with hormones in order to see if they are able to produce viable oocytes. As described in 3.2.4.2.4, females were sacrificed 13-14 h after hCG injection. Oocytes from 8 females were placed in two or three fertilization plates. This procedure was carried out for wild type females, here referred as *Hmox1*$^{+/+}$ (n = 8) and for knockout (*Hmox1*$^{-/-}$) females (n = 8). Interestingly, the number of oocytes obtained differed greatly between the *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ females, although they were treated under the same conditions and injected with the same lot of hormones. Age differences between the animals can not play a role since the animals used were age-mated. The total number of oocytes obtained from the wild type females (n=8) was **208**, while the number obtained from the *Hmox1*$^{-/-}$ females (n=8)

was **137**. This difference is depicted in Fig. 52A. A similar graphic was obtained when calculating the number of oocytes per female (calculated taking into account the average of oocytes/female in each plate), as can be seen in Fig. 52B.

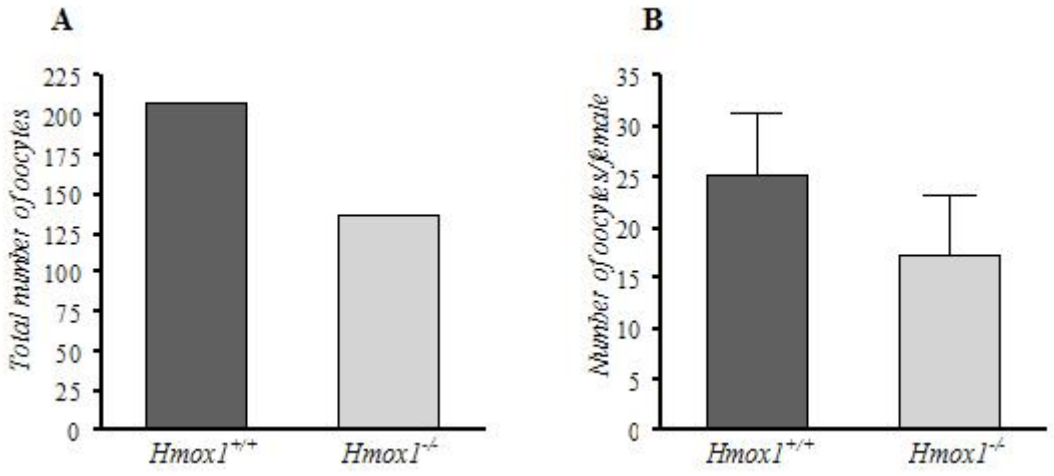


Fig. 52 : *Hmox1*$^{-/-}$ females produce less oocytes than *Hmox1*$^{+/+}$ after hormonal stimulation.

A) Total number of oocytes (n=8 wild type females; n=8 Hmox1$^{-/-}$ females); B) Number of oocytes per female.

Since the procedure of oocyte collection from the oviduct has to be performed very quickly and in a very short period of time (less than 5 minutes per female), and considering that newly ovulated oocytes are surrounded by cumulus cells, it was not possible to count the number of oocytes obtained per female as they were retreated from the oviducts. Once the oocytes were washed and liberated from cumulus cells, it was possible to determine the exact number of oocytes per fertilization plate. As the number of oocytes obtained from the *Hmox1*$^{-/-}$ females was less than the ones from the *Hmox1*$^{+/+}$ females, their fertilization was performed only in two fertilization plates instead of three. For this reason, it was not possible to determine of the difference in the number of oocytes was statistically significant.

4.5.2.2 Analysis of the follicle development in the ovaries from *Hmox1*$^{-/-}$ and *Hmox1*$^{+/+}$ females

In order to analyze the origin of the difference observed in the oocyte number between *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ females, the ovaries of the females used as oocytes donors *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$) where analyzed histologically. Differences in the ovaries due to different sexual maturity between *Hmox1*$^{-/-}$ and *Hmox1*$^{+/+}$ can be discarded since, as mentioned before, the animals used for this study were age-mated.

Histological analysis of the ovaries of females from both groups showed that, even though ovaries from both types of females showed similar morphology, they presented differences regarding their follicle development. In order to quantify these observations, the number of follicles in different stages of maturation was analyzed under the microscope in all samples obtained (in both ovaries from each animal, counting at least three slides per ovary). As depicted in Fig. 53, the total number of oocytes per ovary is similar between both types of females.

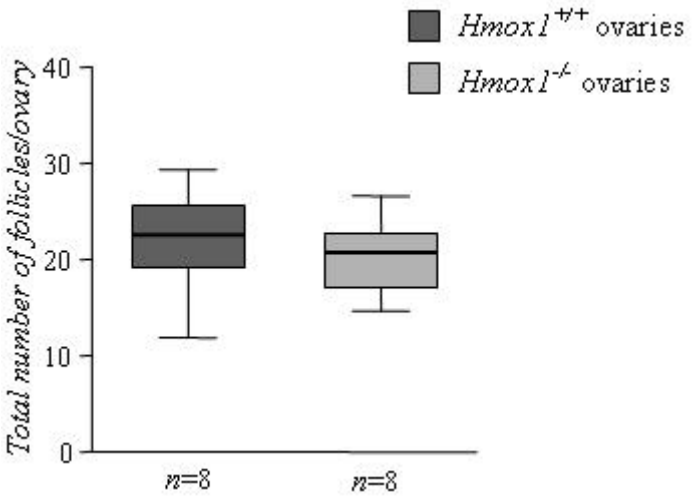


Fig. 53 : Ovaries from *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ females present the same total number of follicles as analyzed under light microscope.

Data are represented by medians ± 75% quartiles. No significant differences were found between the groups Mann-Whitney U-test between both groups.

However, when analyzing the number of follicles in the different maturation stages (Fig 55), some differences were found. Fig 54 is showing an example of follicles in different maturation stages. As it can be observed in Fig. 55, *Hmox1*$^{-/-}$ ovaries show similar number of secondary follicles as *Hmox1*$^{+/+}$ ovaries. The number of primordial, primary and mature follicles differs slightly between both groups, but these differences were not significant. However, when analyzing the number of *corpus luteum* between both groups, *Hmox1*$^{-/-}$ ovaries showed a significant diminution when compared to *Hmox1*$^{+/+}$ ovaries. It is important to mention that the total number of follicle per ovaries does not differ between the groups (Fig. 54), pointing out that the difference in the number of *corpus luteum* account are compensated by a slight

augmentation in the number of primordial and secondary follicles in *Hmox1*$^{-/-}$ ovaries when compared to *Hmox1*$^{+/+}$ ovaries.

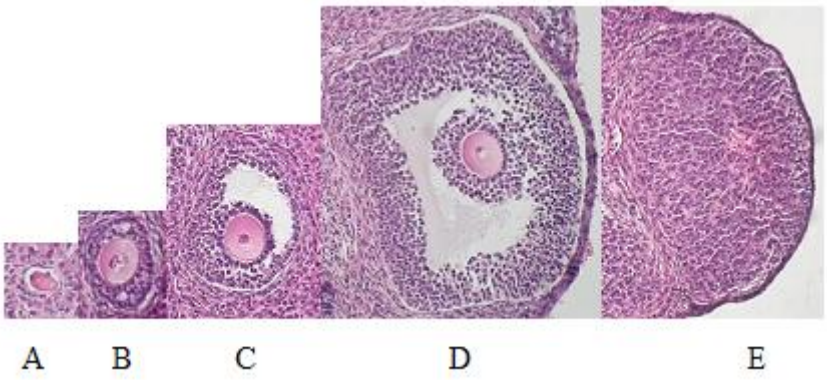


Fig. 54 : Representative pictures of HE staining showing the different stages of follicle maturation as seen under light microscope.

Pictures were taken with a 20X magnification. The differences in the size of the follicles showed in these pictures are in accordance with the difference in sizes observed in the samples.

A) Primordial follicle

B) Primary follicle

C) Secondary follicle

D) Mature follicle

E) Corpus luteum

The significant diminution in the number of *corpus luteum* as shown in Fig. 55 is also in accordance with the fact that *Hmox1*$^{-/-}$ females produced less oocytes than *Hmox1*$^{+/+}$ females (Fig. 52), because the *corpus luteum* is the structure remaining after the release of the oocyte.

Taken all these data together, it is tempting to speculate that *Hmox1*$^{-/-}$ females react differently than *Hmox1*$^{+/+}$ females to hormones, since the follicle development of the ovaries from both differ greatly, even though both groups showed similar number of oocytes in the ovaries. This difference in the response to hormonal treatment may also account for the difference found in the number of oocytes secreted by *Hmox1*$^{-/-}$ and *Hmox1*$^{+/+}$ females. Since the difference in the number of *corpus luteum* between both groups is significant, it is tempting to speculate that the difference in the number of oocytes showed in Fig. 52 may also be significant.

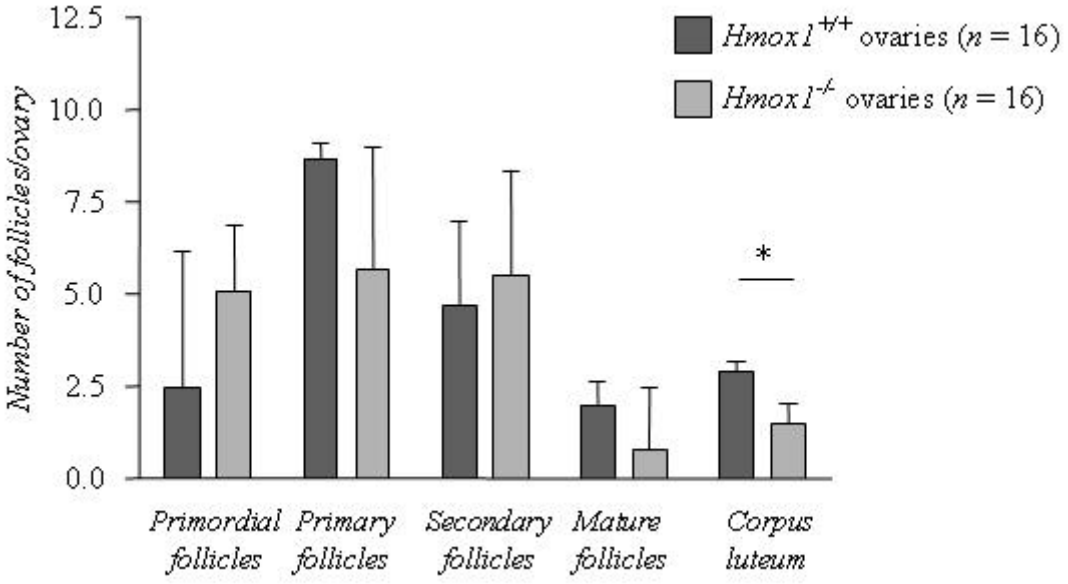


Fig. 55 : Different follicle development in ovaries from *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ females after hormone stimulation as analyzed by light microscopy.

Data are represented by median & interquartile range. Significant differences between groups are indicated as asterisks above lines. *p≤ 0.05 as analyzed by the Mann-Whitney U-test between both groups. For each animal, the mean value of follicles in different stages was calculated analyzing at least 3 slides for each ovary and used as number of follicles per ovary. For each group, 8 animals were analyzed.

4.5.2.3 Assessment of the fertilization rate after *In Vitro* Fertilization (IVF)

Since *Hmox1*$^{-/-}$ do not yield progeny, it was imperative to determine whether this problem could be due to an impaired fertilization. Briefly, *Hmox1*$^{-/-}$ oocytes were *in vitro* fertilized with *Hmox1*$^{-/-}$ sperm whereas *Hmox1*$^{+/+}$ oocytes were *in vitro* fertilized with *Hmox1*$^{+/+}$ sperm. The fertilization rate was assessed by counting the number of oocytes in two-cell stage, and the percentage of fertilization was calculated taking into account the initial number of oocytes. As can be observed in Fig. 56, a statistically significant difference in the fertilization rate of *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ oocytes was obtained.

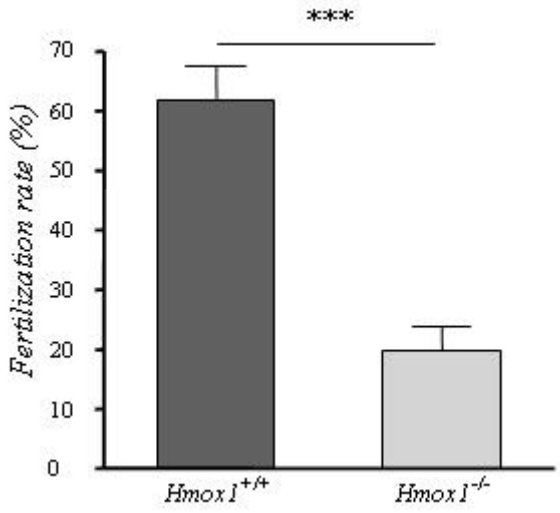


Fig. 56: *Hmox1*$^{-/-}$ oocytes are poorly fertilized with *Hmox1*$^{-/-}$ sperm when compared to *Hmox1*$^{+/+}$ oocytes with *Hmox1*$^{+/+}$ sperm compared to *Hmox1*$^{+/+}$ oocytes by counting the number of oocytes in two cell stage.

Percentages of fertilization rate of Hmox1$^{+/+}$ and Hmox$^{-/-}$ oocytes. The results are expressed as the mean of the fertilization rate obtained in each fertilization plate. ***$p \leq 0.0001$ as analyzed by the Fisher´s exact test between both groups comparing the total number of fertilized and unfertilized oocytes.

A high percentage (**61.70%**) of the *Hmox1*$^{+/+}$ oocytes were successfully fertilized *in vitro*, whereas only **19.78%** of the *Hmox1*$^{-/-}$ oocytes could be fertilized using the exact same conditions (Fig. 56).

The percentage of fertilization obtained for *Hmox1*$^{+/+}$ oocytes was in accordance with the percentages normally obtained using this methodology. The percentage of fertilization of *Hmox1*$^{-/-}$ was extremely low, even though the conditions of fertilization were identical in both cases. The statistically significant difference obtained in the fertilization rates between *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ oocytes is outstanding, since works claiming the importance of other molecules in fertilization present a difference in percentage of 14.1% between wild type and knockout oocytes (Hefler and Gregg, 2002), whereas in this work the difference was as big as 41.92%. The data obtained here clearly shows that deficiency in *Hmox1* has a strong effect in fertilization and opens up an unexpected role of HO-1 in the reproductive field.

4.5.3 Assessment of the implantation capability after embryo transfer

Another possible explanation for the lack of progeny of *Hmox1*$^{-/-}$ could be related to implantation problems beside the impaired fertilization ability of the oocytes and sperm. In order to test this hypothesis, the embryos in two-cell stage obtained by *in vitro* fertilization were transferred into recipient females. The recipient females were hormonally treated and mated with vasectomised males in order to assure that they were in a receptive phase. Only females showing plug with the vasectomised males on the day of the transfer were used for this part of the study.

In order to test whether *Hmox1* is necessary in the mother, in the fetus or in both for implantation to occur, *Hmox1*$^{-/-}$ oocytes were transferred to a *Hmox1*$^{+/+}$ as well as to a *Hmox1*$^{-/-}$ recipient female. Additionally, *Hmox1*$^{+/+}$ oocytes were transferred either to a *Hmox*$^{-/-}$ or to a *Hmox1*$^{+/+}$ female. In this context, it was also interesting to analyze the participation of T cells in HO-1-dependent impaired pregnancies. As we know from a personal communication, the mating of *Hmox1*$^{+/-}$ SCID animals gives more *Hmox1*$^{-/-}$ progeny mice than the mating of *Hmox1*$^{+/-}$ immune competent animals (personal communication of Prof. Soares). Therefore, we also tested whether *Hmox1*$^{+/+}$ are able to implant in SCID *Hmox1*$^{-/-}$ recipient females.

Results for the transfer of *Hmox1*$^{+/+}$ oocytes are schematized in Fig. 57 and in Fig. 58 for the transfer of *Hmox1*$^{-/-}$ oocytes.

Two *Hmox-1*$^{-/-}$ females and two SCID/*Hmox1*$^{-/-}$ females received 10 two-cell stage *Hmox1*$^{+/+}$ embryos in each uterine horn. One *Hmox1*$^{+/+}$ female and one SCID *Hmox1*$^{+/+}$ female received as well 10 two-cell stage embryo in each uterine horn.

Unfortunately, one of the *Hmox1*$^{-/-}$ females died two days after the embryo transfer due to unknown reasons. The other *Hmox1*$^{-/-}$ female was sacrificed on day 14 of pregnancy, and showed no sign of embryo implantation. The uterus was neither swallowed nor was it different from the uterus of a virgin female.

One of SCID *Hmox1*$^{-/-}$ females showed no visible implantations after transfer of *Hmox1*$^{+/+}$ oocytes, but inflamed uterus. However, the other SCID *Hmox1*$^{-/-}$ female presented two implantations after the transfer of *Hmox1*$^{+/+}$ oocytes. It is possible that the first recipient female rejected the embryos after implantation, therefore the inflamed uterus. Unfortunately, the SCID *Hmox1*$^{+/+}$ female, which was supposed to serve as a control, showed no sign of visible implantations.

Hmox1$^{+/+}$ *oocytes*

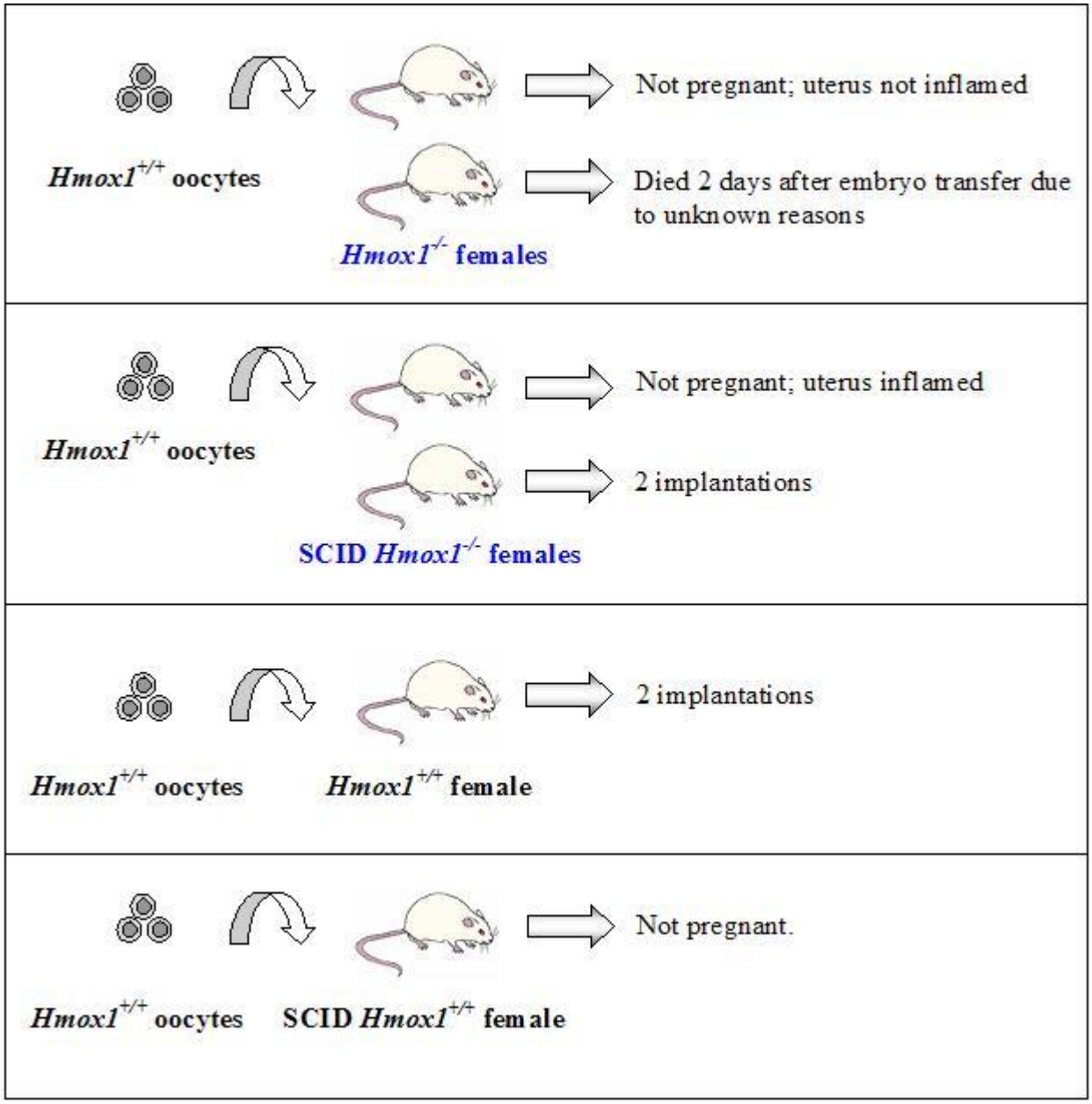


Fig. 57 : *Hmox1*$^{+/+}$ embryos were not able to implant in *Hmox1*$^{-/-}$ uteruses

Implantation success was assessed 14 days after embryo transfer.

The *Hmox1*$^{+/+}$ female showed 2 implantations after transfer of *Hmox1*$^{+/+}$ oocytes.

These results suggest that in immune competent animals, the lack of *Hmox1* can lead to implantation failure, as *Hmox1*$^{+/+}$ oocytes were not able to implant in *Hmox1*$^{-/-}$ uterus while these oocytes were able to implant in *Hmox1*$^{+/+}$ uterus. The fact that these embryos were able to implant in SCID animals lacking *Hmox1* suggests that in immune competent animals,

HO-1 is necessary to counteract the inflammation in the uterus due to the presence of immune cells of maternal origin, which may attack paternal antigens.

Hmox1$^{-/-}$ *oocytes*

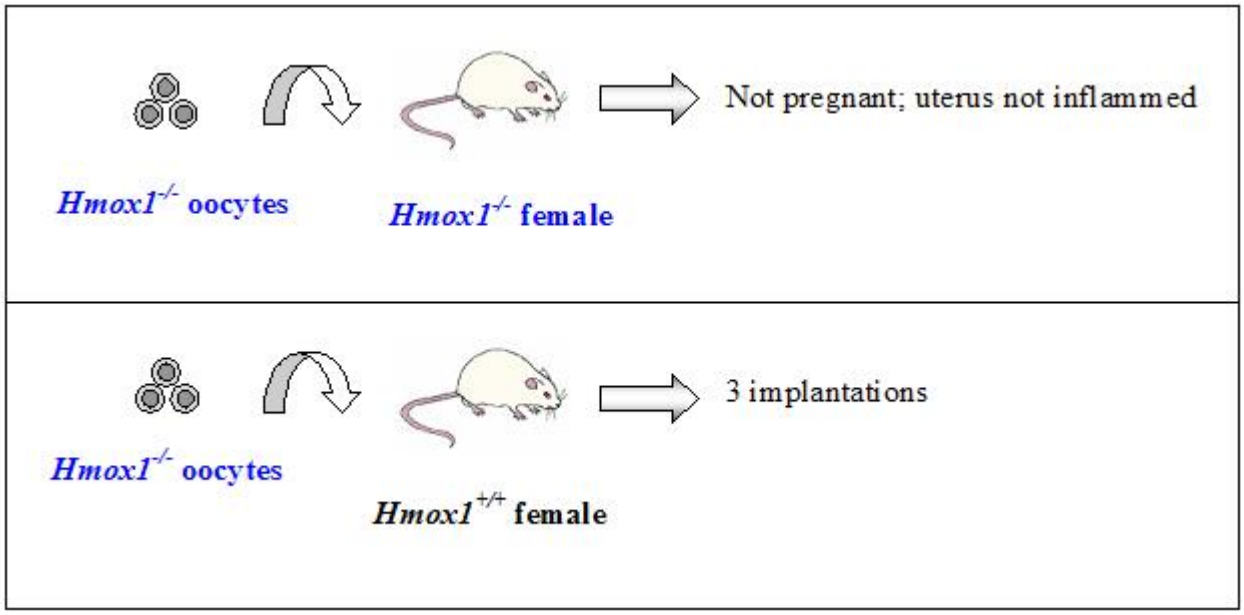


Fig. 58 : *Hmox1*$^{-/-}$ embryos are able to implant in *Hmox1*$^{+/+}$ uterus

Implantation success was assessed 14 days after embryo transfer.

Due to the low number of *Hmox1*$^{-/-}$ fertilized oocytes, the transfer could be only performed into two females (one *Hmox1*$^{+/+}$ and one *Hmox1*$^{-/-}$).

The *Hmox1*$^{+/+}$ female implanted three *Hmox1*$^{-/-}$ embryos, showing that *Hmox1*$^{-/-}$ can normally implant if the uterus express HO-1. However, the *Hmox1*$^{-/-}$ female showed no implantation. The uterus was nor inflamed or different from the uterus of a virgin female. This suggests again need of uterine HO-1 expression for implantation to occur.

All these data together suggest that HO-1 is essential in the female uterus for implantation to occur beside the already analyzed role of HO-1 in prevention of rejection.

5 Discussion

Mammalian pregnancy is a parabiotic union of two genetically different individuals, the fetus and the mother. At the feto-maternal interface, inflammatory processes can occur due to the invasion of microorganisms, but also due to an immune reaction against alloantigens on the fetus or trophoblast. Many studies in animals and humans indicate that some degree of systemic or uterine inflammation is necessary for both normal implantation and pregnancy, but if this inflammation becomes too excessive it can cause pregnancy complications such as abortion (reviewed in Christiansen *et al.*, 2006). Many changes in uterine physiology that occur following the implantation of the blastocyst resemble classical inflammation at the mucosal surfaces of the female reproductive tract (Mellor and Munn, 2000). One of the first works showing the similarity between implantation of the blastocyst and inflammation was the demonstration in rats by Psychoyos (Psychoyos, 1976) that areas of the uterus containing implanting blastocysts stained blue following the intravenous injection of Pontamine Sky Blue. This demonstrated increased blood flow and permeability of the vessels supplying implantation sites, a response known to be the basis of the main early stages of inflammation (Finn, 1986). As pointed out in previous reports, heme oxygenase-1 (HO-1) plays a key role in inflammatory processes (rewieved in Ryter *et al*., 2006). Viewing pregnancy mainly as an inflammatory process had led us to the idea that HO-1 may play an important role in pregnancy. Previous works from our group and from others have shown that HO-1 as well as HO-2 are expressed in different trophoblast cells (Lyall *et al.*, 2000; Barber *et al.*, 2001; Zenclussen *et al.*, 2002b and 2003b), and diminished levels of both can be found in immunological pregnancy complications in humans as well as in animals models (Zenclussen *et al.*, 2002b and 2003b). This suggests that the heme oxygenase system is necessary at the feto-maternal interface, and that its degree of expression may be related to the success or failure of pregnancy.

Pregnancy is viewed by many from a histoincompatibility point of view. Many propose the histoincompatibility between maternal and paternal antigens that provokes a semi-allogeneic fetus as one of the main causes for fetal acceptance or rejection. In this regard, it is thought that an exacerbated immunological reaction towards the fetus may provoke an immunological misbalance that would lead to fetal rejection. According to this, fetal rejection mechanisms would resemble the mechanisms involved in the rejection of an allograft. In the transplantation field, HO-1 has also been shown by others to play an important role and to have beneficial effects when up-regulated (Soares *et al.*, 1998; Coito *et al.*, 2002; Tullius *et al.*, 2002).

Considering the important role of HO-1 in inflammatory processes as well as in transplantation, and regarding the resemblance of both entities to different stages of pregnancy, the main aim of this work was to analyze the role of HO-1 in the different processes related to pregnancy by means of functional studies employing *in vivo* as well as *in vitro* models. In the first part of the work we aimed to clarify whether a specific up-regulation of HO-1 may be able to rescue mice from spontaneous abortion using a well established abortion model. The HO-1 up-regulation was performed by applying an adenoviral vector containing HO-1. We further aimed to obtain T cells over-expressing HO-1 as a therapeutic tool. In the second part of this thesis, considering that trophoblasts are important sources of HO-1, we aimed to clarify whether HO-1 is indispensable in the process of trophoblast survival and differentiation into giant cells. For this, we used a trophoblast stem cell line which differentiates into trophoblast giant cells in order to *in vitro* resemble the process of placentation. In the last part of this work, mice deficient in *Hmox1* were used for clarifying the role of HO-1 in earlier stages of pregnancy, namely fertilization and ovarian follicle development. Besides, different mating combinations of mice partially or totally deficient in *Hmox1* were used to analyze the pregnancy outcome, to try to clarify if HO-1 is necessary in the female, in the male or in both for successful pregnancy to occur. All these points are discussed below.

Role of HO-1 in immunological spontaneous abortion

Previous studies from our group and from others support the concept that, during pregnancy, different types of trophoblast cells are important sources of HOs (Ihara *et al.*, 1998; Barber *et al.*, 2001; Zenclussen *et al.*, 2003; Zenclussen *et al.*, 2005) and would participate in the catabolism of the heme protein, avoiding accumulation or recirculation of free heme, which could be extremely toxic for the mother and for the fetus. HO-1 and HO-2 down-regulation has a high potential to be harmful at the feto-maternal interface, since high amounts of free heme readily incorporate into endothelial cells, leading to oxidative injury and enhanced adhesion molecule expression (Balla *et al.*, 1991; Wagener *et al.*, 1997; Vachharajani *et al.*, 2000), allowing the migration of inflammatory lymphocytes into the feto-maternal interface. Interestingly, previous works have reported down-regulated HO-1 and HO-2 levels at the feto-maternal interface from mice undergoing abortion (Zenclussen *et al*., 2005). Accordingly, low

HO-1 and HO-2 levels were found in placental tissues from patients undergoing abortion when compared to normal pregnant patients (Zenclussen *et al*., 2003).

Although the presence and the putative protective role of HO-1 during pregnancy have been previously reported (Ihara *et al*., 1998; Barber *et al*., 2001; Zenclussen *et al*., 2003; Zenclussen *et al*., 2005c), it was still unknown if an up-regulation of HO-1 around implantation window would have beneficial effects on pregnancy outcome. During the preparation of this thesis, it was shown by another work from our group that an up-regulation of HO-1 through chemical induction by means of CoPPIX can diminish the abortion rate (Sollwedel *et al.*, 2005). Most interestingly, the down-regulation of HO-1 through ZnPPIX during implantation boosted abortion up to 60%, suggesting that HO-1 plays an important role in avoiding fetal rejection (Sollwedel *et al.*, 2005). However, the mechanisms by which HO-1 exerted its action were not clear in the employed onset, since many of the effects observed can be accounted to secundary effects of the chemical compound CoPPIX and in many cases were similar to the effects observed by ZnPPIX application.

In the first part of the present work, the CBA/J x DBA/2J combination, an established mouse model to investigate immunological pathways leading to spontaneous abortion was employed (Chaouat *et al.*, 1988), to analyze the effect of HO-1 up-regulation on pregnancy outcome. Pregnant mice were injected with an adenovirus coding for HO-1 and the reporter gene GFP. As indicated by similar abortion rates to the PBS-treated abortion-prone animals, the injection of a vector containing EGFP alone did not provoke any deleterious effects in the pregnancy outcome. We could show that the application of viral vectors containing putative protective genes provides a useful and valid system to glance at their effects on pregnancy outcome in this experimental model.

Here, it could be shown for the first time that a *specific* increase in the systemic HO-1 expression following gene transfer of 1.10^5 PFU AdHO-1/GFP improves pregnancy outcome in a murine model of abortion by diminishing fetal rejection rate. The avoidance of accumulated free heme might be one main reason for the success of the therapy, since an excess of free heme can cause cell damage and conduce to tissue injuries by the formation of reactive oxygen species (Vercellotti *et al*., 1994). Nevertheless, mice receiving 1.10^8 PFU AdHO-1/GFP showed no significant diminution in the abortion rate. One has to keep in mind that all the heme-degradation products are potentially toxic, depending on the generated amounts and the microenvironment (Wagener *et al*., 2003). Too high HO-1 expression may cause tissue injury by generating high levels of iron or resulting in bilirubinemia (Suttner and Dennery, 1999;

Tyrrell, 1999). Interestingly, mice receiving high doses of the AdHO-1/GFP vector showed icteric sera and amniotic liquid, suggesting a too high HO-1 expression and subsequently hyperbilirubinemia and toxicity against the fetus.

The fact that no significant differences regarding HO-1 levels were found at mRNA or protein levels after AdHO-1/GFP treatment may be related to the time point chosen for analysis (9 days after adenovirus injection). It is known that adenoviruses are cleared between 5 and 20 days post-infection (Dai *et al.*, 1995), due to the strong immune that they generate in the host. Having sacrificed the animals 9 days after the injection implies the possibility that most of the transduced cells were already cleared and therefore no major differences regarding HO-1 expression could be found. It would have been interesting to sacrifice the animals 2 or 3 days after injection of the adenovirus to analyze the pattern of HO-1 expression in different organs. However, if that would have been the case, it would have been impossible to analyze the pregnancy outcome, since at day 8 of pregnancy it is still impossible to differentiate between a healthy fetus and a resorption. However, some transduced cells were found in some of the treated animals. As it was shown by fluorescence microscopy, trophoblast cells were efficiently transduced by the adenovirus, whereas no GFP expression was found in fetal tissue. This is in accordance with previous reports pointing out that fetal membranes may act as a barrier, which may naturally prevent adenoviral particles from passing between embryonic cavities (Laurema *et al.* 2004). On the other side, when analyzing GFP expression by PCR, most of the GFP expression was found in the maternal liver. This is due to the fact that the liver sequesters the majority of systemically administered Ad particles via hepatic macrophages (Kupffer cells) (Glasgow *et al.*, 2006) and is in accordance with the already described hepatic tropism of adenoviruses. However, it was surprising to find comparable levels of GFP DNA expression in the fetuses and placentas. The fact that no GFP expression was observed by fluorescence microscopy in the fetuses may suggest that adenoviral vectors may have been able to infect some cells of the fetus, but the protein was not expressed. Interestingly, a report by Everett *et al.* analyzing the persistence of adenoviral vectors in different strains of animals have shown that despite the persistence of the vector genome in the liver, a precipitous loss of LacZ protein expression was found. One of their explanations was that the differential elimination of transgene expression may have been due to a strain-specific differential down-regulation of the CMV enhancer/promoter element utilized to drive the LacZ gene in their Ad vector (Everett *et al.*, 2004). One alternative speculation for the promoter shutdown was a differential rate of down-regulation of transcriptional factors induced by adenoviral infection. It is tempting to speculate that in the work presented in this thesis, a differential down-

regulation of the CMV-promoter took place in the fetuses, making the GFP protein not expressed in this tissue. The GFP expression found by PCR can also arise from fetal skin, since Senoo and co-workers have also been able to find low adenoviral expression in fetal skin (Senoo *et al*., 2000).

Regarding the mechanisms by which the adenoviral vector containing HO-1 is pregnancy-protective in this model, one of them seems to be a Th2 polarization. In this work, augmented levels of IL-4 as well as diminished levels of IFN-γ were found systemically and locally after application of AdHO-1/GFP. The "Th1/Th2/Th3 paradigm" proposes that a balance between Th1 and Th2/Th3 cytokines is critical for pregnancy development. Th2 and Th3 type cytokines such as IL-4, IL-10 (Th2) and TGF-β (Th3) were proposed to favour the maintenance of mammalian pregnancy (Lin *et al.*, 1993; Chaouat *et al.*, 1995), whereas the excessive production of Th1 cytokines (IL-2, IFN-γ, TNF-α) would mediate the rejection of the fetus at the feto-maternal interface (Lin *et al.*, 1993; Raghupathy, 1997). Down-regulated HO levels were related with increased Th1 levels in transplantation immunology (Woo *et al*., 2000) and the up-regulation of HO-1 by CoPPIX diminished Th1-cytokines levels while being successful in allowing graft acceptance (Woo *et al.*, 2000). Here, we found diminished IL-4/IFN-γ (Th2/Th1) ratio at the fetal-maternal interface from abortion-prone mice, confirming previous results of the group (Zenclussen *et al.*, 2005c). Treatment of mice undergoing abortion and presenting elevated Th1 levels with 1.10^5 PFU AdHO-1/GFP provoked an augmentation in Th2 secretion accompanied by a diminution in the Th1 production, as shown by an augmented IL-4/IFN-γ ratio when compared to animals treated with PBS or the control vector. Here, enhanced Th2/Th1 ratio was found in spleen as well as in decidual lymphocytes from mice in which the therapy was successful in avoiding fetal rejection, suggesting a local and systemic effect of the therapy on the Th2/Th1 secretion. It is tempting to speculate that the up-regulation of HO-1 protects the tissues by stimulating cells to shift their cytokine production to a Th2 profile. Regarding the expression of IL-10 and TNF-α, a tendency towards an augmentation of the IL-10/TNF-α ratio was found in spleen lymphocytes from mice treated with AdHO-1/GFP. In decidual lymphocytes, however, normal pregnant mice presented a diminished IL-10/TNF-α ratio when compared to the abortion prone-group. In accordance with the levels found in normal pregnant mice, decidual lymphocytes from mice receiving AdHO-1/GFP presented a diminished IL-10/TNF-α ratio when compared to the abortion-prone mice group. Nevertheless the differences were not significant for any of the cases (spleen and decidual lymphocytes). Even though the results were not as expected, the IL-10/TNF-α levels in

decidual cells of the group receiving the AdHO-1/GFP behaved in the same way as the decidual cells from normal pregnant animals. Our data on the influence of HO-1 on the Th1/Th2 ratio are in agreement with data by Woo and co-workers showing that an up-regulation of HO-1 by CoPPIX diminished the IFN-γ production in a graft-versus-host disease model (Woo *et al*., 2000).

To analyze whether the Th2 augmentation responds to a major number of Th2 lymphocytes migrating to the fetal-maternal interface or to a polarization of lymphocytes to Th2, lymphocyte infiltration was measured in placental and decidual tissue. In placental tissue, no differences were found between the groups, suggesting that HO-1 application did not lead to an augmented lymphocyte infiltration into the fetal component of the feto-maternal interface. In decidual tissue, however, a significant augmentation of the CD3 mRNA expression was found in the group of mice receiving the AdHO-1/GFP vector. This suggests an augmented infiltration into the maternal part of the feto-maternal interface. Considering the already mentioned Th2 shift, it is tempting to speculate that these lymphocytes were Th2.

We next analyzed whether HO-1 application has any effect on a specific lymphocyte subpopulation, namely regulatory T cells (Treg) as Treg were proposed to be important for pregnancy maintenance (Zenclussen, 2005; Saito *et al.*, 2005). Interestingly, it has been proposed that it may be a relationship between Treg and HO-1 (Pae *et al.*, 2003; Choi *et al.*, 2005), whereas others do not find any relation (Zelenay *et al*., 2007). We have shown that mice that were rescued from abortion by the adoptive transfer of Treg presented augmented levels of HO-1 at the feto-maternal interface (Zenclussen *et al.*, 2006). This suggests that Treg are able to induce HO-1 expression at the feto-maternal interface. On the other hand, we could also show that HO-1 up-regulation by means of CoPPIX augmented the levels of the Treg marker Neuropilin-1 (Sollwedel *et al*., 2005), suggesting a bi-directional relationship between HO-1 and Tregs in pregnancy. In this work, no relation could be found between HO-1 and Treg as the application of an adenoviral vector containing HO-1 did not modify the levels of the markers analyzed (Foxp3 and TGF-β).

Since HO-1 is known to have anti-apoptotic properties (Soares *et al.*, 1998), the possible influence of HO-1 up regulation on apoptosis-related events at the fetal-maternal interface was investigated. AdHO-1 treatment could slightly down-regulate the placental caspase-3 activity when compared to abortion-prone mice treated with PBS or with a control vector, suggesting diminished apoptotic activity at the feto-maternal interface. Moreover, the TUNEL staining allowed us to confirm a significant diminution in the number of apoptotic cells in placenta

from mice receiving low doses of AdHO-1/GFP when compared to PBS- or EGFP-treated abortion-prone mice, confirming therefore the anti-apoptotic effect of the HO-1-therapy at the feto-maternal interface. Since the caspase-3 activity was not statistically diminished after AdHO-1 therapy, while the number of apoptotic cells was, it is tempting to speculate that apoptosis in this system is not caspase-3 dependent. Other works have already pointed out that the anti-apoptotic effects of HO-1 may act through a caspase-3 independent pathway (Lang *et al.*, 2005). The anti-apoptotic effect observed in our model is also in accordance with anti-apoptotic effects of HO-1 observed in transplantation (Coito *et al.*, 2002; Braudeau *et al.*, 2004). Interestingly, a work of Redaelli and co-workers in transplantation showed like we did that HO-1 had anti-apoptotic effects in a model of liver graft, and this was associated with significantly lower number of apoptotic cells (detected by TUNEL) and slightly diminished levels of caspase-3 (Redaelli *et al.*, 2002). The work of Coito and co-workers also suggest that HO-1 may also act by up-regulating anti-apoptotic molecules like Bcl-2 and Bag-1 (Coito *et al.*, 2002). Accordingly, we found augmented mRNA levels of the anti-apoptotic molecule Bag-1 which reinforces an anti-apoptotic/cytoprotection hypothesis. This data strongly suggest that Bag-1 over expression following HO-1 treatment is directly implied in the avoidance of abortion. As Bag-1 mRNA is not significantly down-regulated in the abortion-prone combination compared to the normal one, the finding that AdHO-1 induces Bag-1 expression may be specific to gene over-expression. In fact, Bag-1 was already described to be augmented after up-regulation of HO-1 by gene therapy in tolerant grafts (Coito *et al.*, 2002). Since the first demonstration of Bag-1 as an anti-apoptotic molecule (Takayama *et al.*, 1995), Bag-1 over-expression has been shown to inhibit apoptosis induced by a wide range of inducers in various cell types (reviewed in Townsed *et al.*, 2003). The mechanisms accounting for the effects of Bag-1 on apoptosis induced by several stimuli are not fully characterized yet. One potential target is the Bcl-2 protein, a Bag-1 interaction partner (Townsed *et al.*, 2003). Thus, it is possible that the broad effects of Bag-1 derive from its targeting of this key Bcl-2-regulated mitochondrial apoptosis checkpoint. Further, Bag-1 interacts with the HSC70 and HSP70 heat shock proteins and promotes cell survival by coordinating the function of these chaperones with the proteasome to facilitate protein degradation (Townsed *et al.*, 2004). Interestingly, Townsend *et al.* have demonstrated that Bag-1 has a novel cytoprotective function, e.g. protecting cardiac myocytes from apoptosis induced by simulated ischemia/reperfusion, mediated via association with HSC70/HSP70, which is critical upon cytoplasmic localization but independent of the BAG-1 ubiquitin-like domain (Townsed *et al.*, 2004). Accordingly to our data, we speculate that Bag-1 is mediating fetal tissue protection. This data is in accor-

dance with other data from our group, where the protective effect of HO-1 through CoPPIX application was also related to augmented levels of Bag-1 at the feto-maternal interface (Sollwedel *et al.*, 2005).

Heme oxygenase-1 has also been described to be involved in angiogenesis. In this regard, Dulak and co-workers have shown that these angiogenic effects are related to the modulation of vascular endothelial growth factor (VEGF) synthesis by HO-1 activity (Dulak *et al.*, 2002). In pregnancy, the endometrium, decidua and placenta are sources rich if angiogenic growth factors (Zygmunt *et al.*, 2003), and VEGF production by the trophoblasts during pregnancy has paracrine effects on maternal endothelial cells resulting in vascular remodelling and/or permeability within the microcirculation. Besides, trophoblast derived VEGF acts by increasing the efficiency of vascular-mediated exchange between the mother and the fetus. In this work, an up-regulation of HO-1 led to a slight augmentation in the number of VEGF positive cells in placental tissue, suggesting that HO-1 may have had angiogenic effects in this model. Knowing the relationship between VEGF and HO-1 expression, it is tempting to speculate that HO-1 up-regulation during implantation may have augmented VEGF expression within the feto-maternal interface, favouring a proper vasculogenesis and angiogenesis that would lead to a proper implantation of the fetuses and consequently to an avoidance of fetal rejection.

The protective effect of up-regulated HO-1 levels in our treatment could be also related to increased carbon monoxide (CO) production (Sato *et al*., 2001). Although CO was considered for years a very dangerous gas (Johnson *et al*., 1999), it is now clear that CO can reduce inflammatory responses by inhibiting pro-inflammatory genes and by augmenting anti-inflammatory cytokine production (Otterbein and Choi, 2000; Sarady *et al.*, 2002; Song *et al.*, 2003). Interestingly, inhibition of HO-1 by SnPPIX did not conduce to graft rejection if the mice were simultaneously exposed to CO. Besides, both, gene transfer of HO-1 or CO application inhibited chronic graft rejection (Chauveau *et al.*, 2002). These data indicate a crucial role for CO in transplant acceptance and suggest that the HO-1-related beneficial effects are related to CO production. Data obtained in our group by Ivonne Wollenberg (Diploma thesis), showed that exposure to 250 p.p.m. of CO during implantation (days 4 and 5 of pregnancy) could completely avoid abortion in the CBA/J x DBA/2J model, indicating a beneficial effect of CO during pregnancy. However, CO inhalation during the whole experiment (days 0-13 of pregnancy) led to detrimental effects on the fetuses and the mother, and even to unsuccesful pregnancies (no viable litters). This indicates that CO in specific amounts

and in specific time points of pregnancy can be protective, and that may also suggest that the effects seen in this approach may be due to the effects of CO. If biliverdin/bilirubin or free iron were involved in the protective effect of our work is unclear. However, it has been shown in other models that biliverdin can imitate the effects of HO-1 by inducing tolerance to cardiac allografts (Yamashita *et al.*, 2004), suggesting that some of the effects seen by the up-regulation of HO-1 can be explained by augmented biliverdin levels. The third by-product of the HO-1 enzymatic reaction, free iron, is potentially toxic but can give antioxidant protection when coupled with proteins that promote their sequestration or export of the liberated iron. In this regard, ferritin has been also proposed as a cytoprotective molecule and as a contributory mechanism underlying HO-dependent protection (reviewed in Ryter, 2006). It was not found in the literature if an up-regulation of biliverdin or ferritin can be protective in models of pregnancy complications, and it is unknown if in our model the up-regulation of HO-1 acted through its compounds.

Summarizing, the use of an adenoviral vector containing HO-1 allowed us to glance at the mechanisms by which HO-1 would have a protective role during pregnancy. Taken together, our findings point out a protective effect of systemic HO-1 up regulation on pregnancy outcome. A complex picture of regulatory interactions between the HO system and apoptotic and cytokine networks is suggested. These data could further open new opportunities finding novel therapeutic directions in immunological pregnancy failure.

The use of an adenoviral vector over-expressing HO-1 was used in order to elucidate the important role of HO-1 in avoiding fetal rejection, and not intended to be proposed as a therapy. A more therapeutical approach was intended to do using T cells over-expressing HO-1. The use of retroviral vectors, reported by others as being a successful method for the transduction of T cells (Hammer *et al*., 2002; Hori *et al*., 2003), was in our hands not successful enough. One of the reasons why this approach could not be established may be that cells from CBA/J females were poorly stimulated with paternal antigens, since retroviral vectors are known to transduce only dividing cells (Somia and Verma, 2000). The attempt of stimulating cells with anti-CD3 and anti-CD28 did improve the proliferation of these cells, but the transduction efficiency was not significantly improved. The other possibility for the lack of success of this approach may be the vector (pLXSN), since works from our group (Paul Wafula, ongoing PhD thesis) using other retroviral vector (MIGR-1) are currently being successful in transducing CBA/J cells stimulated with anti-CD3 and anti-CD28 (but not stimulated with paternal antigens). Since the maximal transduction efficiency obtained in this approach was less than

8%, it was not feasible to obtain enough amounts of cells to be transferred in sufficient number of animals. For this reason, the approach using protein transfection was performed.

The protein transfection has been used to transfect mainly adherent cells (Morris *et al.*, 2001) and has even been used *in vivo* (Aoshiba *et al*., 2003). However, no report has been found to date about the transfection of murine primary T cells. In the context of this PhD work, a successful transfection has been achieved in $CD4^+$ T cells. The practical convenience of this method relies on the time that is saved when compared to retroviral transduction, and the percentage of efficiency obtained. $CD4^+$ cells have been efficiently transfected using the positive control (80% of transfection), and have been also efficiently transfected with HO-1. When used *in vitro*, however, these cells were not showing any particular effect on the proliferation of cells from a CBA/J female. The effects observed were always comparable to those observed with cells treated with the transfection agent alone (T_{MOCK}). *In vivo* (data not shown) these cells were also not effective, reason why the experiment was not fully completed. One possible explanation for this may be that the protein transfected into the cells is not active. The HO-1 protein used for this approach was commercially available, and the company was not able to inform us whether the protein is active or not. The other possibility may be that $CD4^+$ T cells are not the right target for the transfection of HO-1. Alternative targets for the HO-1 transfection could be dendritic cells, since HO-1 is known to act on dendritic cells by keeping them in an immature state (Chauveau *et al*., 2005).

Role of HO-1 in trophoblast survival and differentiation

After implantation, the trophectoderm surrounding the blastocyst goes on to differentiate into a variety of trophoblast cell subtypes with different functions. Trophoblast stem cells emerge from the polar trophectoderm that overlies the inner cell mass of the blastocyst, and they proliferate in response to close contact to the inner cell mass (Cross, 2005). From the different types of trophoblasts in the mouse, the giant cells are the first to arise, already at the blastocyst stage. These cells exit the mitotic cell cycle and stop dividing, enlarge, and they undergo endoreduplication to become polyploid (MacCauley *et al.*, 1998). The function of trophoblast giant cells is to first mediate the process of implantation and invasion of the conceptus into the uterus. Later they produce several hormones and cytokines to promote local and systemic physiological adaptations in the mother including the regulation of maternal blood flow to the

implantation site, and production of progesterone from the ovary (reviewed in Cross, 2005). Due to the vital importance of the giant cells in the formation of the placenta and in the establishment of a successful pregnancy, the main aim of this part of the study was to analyze *in vitro* whether HO-1 is necessary for trophoblast stem cell differentiation into giant cells.

The Rcho-1 cell line is usually used as an *in vitro* model for studying trophoblast cell differentiation (Faria and Soares, 1991). These cells can be manipulated to proliferate or differentiate into trophoblast giant cells by altering culture conditions. Rcho-1 trophoblast stem cell differentiation recapitulates *in vivo* trophoblast giant cell development, and is a valuable *in vitro* tool for studying the process of trophoblast cell differentiation (Sahgal *et al.*, 2005). So far, HO-1 was reported to be expressed in different trophoblast cells of human and murine placentas (Ihara *et al.*, 1998; Barber *et al.*, 2001; Zenclussen *et al.*, 2003b; and 2005b), and its expression in the murine placenta is normally the highest in giant cells (personal observation). However, no report was found in the literature showing that HO-1 is necessary during the differentiation process of trophoblasts. In the experiment showed in this thesis, the main aim was to analyze whether a down-regulation of HO-1 renders these cells unable to proliferate into giant cells. This would help us understand if HO-1 is really necessary for the differentiation of trophoblast stem cells, and therefore to the proper formation of a placenta when translated into an *in vivo* situation.

Interestingly, the down-regulation of HO-1 expression by ZnPPIX led already to a significantly diminished viability of precursor stem cells, suggesting that HO-1 is necessary even for the survival of trophoblast stem cells. The application of CoPPIX did not lead to significant changes in the viability of Rcho-1 cells, suggesting that the effect seen with ZnPPIX may not be due to the toxic effect of the porphyrin *per se*. When analyzing the ability of these cells to differentiate into giant cells, the application of ZnPPIX led them unable to transform into giant cells, whereas cells treated with CoPPIX did not show any problem in the differentiation process. These results suggest that HO-1 may play an important role in the differentiation process of trophoblast cells. No results have been found in the literature on this regard, making this a novel discovery in the role of HO-1 in formation of placenta.

The fact that mice lacking *Hmox1* do not get pregnant also suggests that without HO-1 no placentation is able to occur.

Role of partial or total loss of HO-1 on the outcome of pregnancy

As already described by others (Poss and Tonegawa, 1997, Yet *et al*., 1999) and as mentioned before, no progeny can be achieved when mating *Hmox1*$^{-/-}$ mice. Interestingly, the mating of heterozygous females and males leads to only 3-10% of knockout progeny, instead of the 25% expected Mendelian rate. Both facts suggest that *Hmox1* plays a very important role in pregnancy, but so far no explanation for this problem in the maintenance of the colony was found in the literature. For this reason, we aimed to analyze the origin of this problem by analyzing if the low progeny of the heterozygous combination is due to rejection of the fetuses or to other reasons. Besides, different combinations of wild type, heterozygous and knockout animals were performed in order to assess whether the problem resides in the females, in the males, or in both.

When females partially deficient in Hmox-1 (*Hmox1*$^{+/-}$) were mated with *Hmox1*$^{-/-}$ males, only 4 out of 11 females that showed insemination plug were indeed pregnant, and showed an abortion rate of 38.7 %. The fact that only 4 out of 11 females were pregnant suggests that *Hmox1*$^{-/-}$ males may present fertilization problems. Similarly, when *Hmox1*$^{+/+}$ females were mated with *Hmox1*$^{-/-}$ males, 3 out of 5 females mated were pregnant, suggesting here that *Hmox1*$^{-/-}$ males may have fertilization problems. However, *Hmox1*$^{+/+}$ males also showed fertilization problems when fertilizing *Hmox1*$^{+/-}$ or *Hmox1*$^{+/+}$ females, since not all females showing insemination plug were pregnant. This suggests that these fertilization problems may not be exclusively associated with the lack of Hmox1 in the male.

Analyzing the situation from the female site, an interesting result when using the heterozygous females was observed, namely that the abortion rate is higher when less HO-1 is present in the male, thus leading to less HO-1 in the F1 tissue (placenta). This was as follows: when heterozygous females were mated with *Hmox1*$^{+/+}$ males, they presented an abortion rate of 18.4 %, with *Hmox1*$^{+/-}$ males the abortion rate was of 22.4 %, whereas with *Hmox1*$^{-/-}$ males the abortion rate increased to 38.7%. This clearly shows a trend towards an augmentation on the abortion rate with a diminution of HO-1 in the system. When *Hmox1*$^{+/+}$ females were mated with all three types of males, they showed very similar abortion rates in all three types of combinations. All these data together shows that, when the female present normal HO-1 levels, the male HO-1 contribution is not relevant for the success of pregnancy. However, when the female is already partially lacking HO-1, the less HO-1 the males are contributing

the more abortion can be observed. This data strongly suggests that HO-1 is very important in the maternal site for a successful pregnancy, whereas its absence in the paternal site may not be as important as like in the maternal site.

No references in the literature were found regarding the necessity of HO-1 in the oocytes or sperm for reproductive processes. For HO-1-related molecules, however, interesting information has been published. It has been pointed out that a low concentration of nitric oxide may have some physiological role in fertilization through the enhancement of sperm´s capacity to bind to the zona pellucida of the oocyte (Kazuo *et al*., 1998). On the other hand, mice deficient in Cox-2, a molecule related to HO-1, are known to have multiple reproduction failures including problems in ovulation, fertilization, implantation and decidualization (Lim *et al.*, 1997). Interestingly, mice deficient in Cox-2 showed a blunted and delayed induction of HO-1 in a model of endotoxemia (Ejima and Perrella, 2004), suggesting altered HO-1 expression in these animals. It is not mentioned in the literature whether HO-1 is involved in the process of fertilization, but considering the relationship of HO-1 with the nitric oxide system and with Cox-2, it is tempting to speculate that the lack of HO-1 leads to impaired or altered expression of NO and/or Cox-2, which in turn leads to infertility. We did not address this point in the present work and this may lead to interesting results and merits further studies. One can also speculate that animals deficient in Cox-2 present fertilization problems an impaired fertility due to altered expression of HO-1, as we clearly show that HO-1 is important in several steps of reproduction. This work clearly opens the possibility of analyzing this process more accurately in future projects.

When performing an allogeneic combination using *Hmox1*$^{+/+}$ C57/BL6 females mated either with *Hmox1*$^{-/-}$ or *Hmox1*$^{+/+}$ BALB/c males, most of the females presenting insemination plug were pregnant. When the male was deficient in *Hmox1*, an 8.6% of abortion was observed, against 0% of abortion obtained with the *Hmox1*$^{+/+}$ males. These percentages of abortion are very similar, and suggest again that the lack of HO-1 in the male does not significantly affect pregnancy outcome when HO-1 is present in the female.

We next genotyped the offsprings (healthy fetuses as well as unviable implantations) in order to analyze whether animals deficient in HO-1 were implanted and rejected, which may explain the low percentage of knockouts in the *Hmox1*$^{+/-}$ x *Hmox1*$^{+/-}$ matings. The analysis of the genotype of the fetuses and resorptions showed that, surprisingly, the mating of *Hmox1*$^{+/-}$ females with *Hmox1*$^{-/-}$ males lead only to heterozygous offspring. This result strongly suggests that *Hmox1*$^{-/-}$ blastocysts were unable to implant or were rejected in a very early time

point and not visible at the time of preparation, since fetuses as well as resorptions were heterozygous.

The mating of *Hmox1*$^{+/-}$ females with *Hmox1*$^{+/-}$ males led to the typical percentage of knockouts obtained from these matings (personal communication from Prof. Soares and personal observation from the maintenance of the colony), and this percentage is lower than the expected Mendelian rate of 25%. Interestingly, the resorptions were again exclusively heterozygous suggesting that the lower percentage of *Hmox1*$^{-/-}$ fetuses obtained from the *Hmox1*$^{+/-}$ x *Hmox1*$^{+/-}$ mating is not due to immunological rejection of these fetuses but to the lack of implantation of the *Hmox1*$^{-/-}$ blastocysts. This can be due to the fact that, as reported by Chen and co-workers in 2005, blastocysts normally up-regulate HO-1 immediately after hatching (Chen *et al.*, 2005), and in *Hmox1* deficient blastocysts this can not occur, but may be compensated by the presence of HO-1 in the maternal uterus. In the case of heterozygous uteruses, the lack of *Hmox1* in the blastocysts may not be always sufficiently compensated by the HO-1 expression from the uterus, and that may explain why so few *Hmox1*$^{-/-}$ blastocysts are efficiently implanted.

Role of HO-1 in early stages of pregnancy (ovulation, fertilization, implantation)

So far, HO-1 in pregnancy was thought to be important for implantation. The data obtained with the IVF now brings new light into the role of HO-1 in pregnancy as we could confirm that *Hmox-1*$^{-/-}$ females are not able to produce as many oocytes as wild type females, indicating that HO-1 is necessary even at very early stages of reproduction. The difference in the oocyte production may be due to differences in the reaction of the *Hmox1*$^{-/-}$ females to hormonal treatment, since ovaries from *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ females showed the same total number of follicles per ovary. The changes that take place on the ovary at the site of follicular rupture are pathophysiological in nature. This local damage induces hemorrhage in the vicinity of the lesion on the surface of the ovary (reviewed in Espey *et al.* 2004). Considering the importance of the up-regulation of HO-1 in inflammatory processes, especially in events related to hemorrhage, it is tempting to speculate that the lack of HO-1 may impair the ovulation process in a way that these animals do not manage to induce the follicular rupture. Without HO-1, the ability to counteract the inflammatory process related to it is missing. In other words, due to the inflammatory nature of ovulation, mature follicles lacking HO-1 may found

it more difficult to induce the rupture of the tissue due the inflammatory nature of the process, and may be then less secreted than follicles expressing HO-1. Interestingly, NO, an inflammatory mediator normally associated with HO-1, has been described to be involved in ovulation. Studies using *eNOS*$^{-/-}$ mice have shown that they have reduced fertility due to impaired ovulatory efficiency, abnormalities in meiotic maturation, increased oocyte apoptosis and altered estrous cyclicity compared to their wild type littermates (Jablonka-Shariff and Olson, 1998; Drazen *et al.*, 1999; Hefler and Gregg, 2002). Studies using *iNOS*$^{-/-}$ mice show that iNOS deficiency does not alter ovulatory capacity, but it may play a role in fertilization (Yang *et al.*, 2005). Furthermore, the cyclooxygenase (COX) pathway, which is responsible for prostaglandin (PG) synthesis, is also important in inflammatory responses, as the HO and NO pathways are. Additionally, they have constitutive and inducible isoforms (McGarry *et al*., 2005), and they are closely related (Ejima and Perrella, 2004). The two enzymes responsible for the synthesis of PGs are COX1 and COX2, being the first constitutive and the second inducible form (Venturini *et al.*, 1998). Although *Cox1*$^{-/-}$ mice have normal fertility, *Cox2*$^{-/-}$ females are infertile and exhibit abnormalities in ovulation due to PG deficiency (Matsumoto *et al.*, 2001). Regarding the HO system, interestingly, *Hmox2*$^{-/-}$ mice do not present reproductive problems (Poss *et al.*, 1995), whereas *Hmox1*$^{-/-}$ do present problems, as shown in the results presented in this thesis. These results open the possibility of further analyze the problem in a more accurate way, and these studies are being currently done in our group.

We then analyzed the ovaries from the females used as oocyte donors in order to analyze whether the lack of HO-1 is associated with morphological changes. Ovaries from both types of females were morphologically similar, but a significant diminution in the number of corpus luteum of *Hmox1*$^{-/-}$ ovaries when compared to *Hmox1*$^{+/+}$ ovaries was obtained. This is in accordance to the lower number of oocytes obtained from *Hmox1*$^{-/-}$ females. As the number of total follicles per ovary was similar when comparing both groups, the difference in the number of corpus luteum was compensated by a slight augmentation in the number of primordial and secondary follicles in *Hmox1*$^{-/-}$ ovaries when compared to *Hmox1*$^{+/+}$ ovaries. It would be very interesting to know if *Hmox1*$^{-/-}$ females can even ovulate without hormonal stimulation. This will be performed in our institute as soon as sufficient amount of knockout females are available. It will be interesting also to know if these females present normal hormonal levels, and if they present normal estrous cyclity.

The oocytes obtained from *Hmox1*$^{-/-}$ females showed additionally a very poor fertilization rate (19.78%), suggesting that *Hmox1* may also be necessary (but not indispensable) for the fertilization. This may also suggest that *Hmox-1*$^{-/-}$ oocytes are not as viable as wild type oocytes, and may be therefore the reason why the mating of mice heterozygous for HO-1 may yield so few *Hmox-1*$^{-/-}$ progeny (around 3-10% instead of the 25% expected Mendelian rate), and why *Hmox-1*$^{-/-}$ animals do not give viable progeny when paired with *Hmox-1*$^{-/-}$ males. In order to finally confirm that the reason as to why *Hmox1*$^{-/-}$ animals are not fertile and *Hmox*$^{+/-}$ do not yield the expected Mendelian rate, current experiments with Hmox1 deficient animals are being carried out. However, taking into account the results from the different combinations, I would speculate that *Hmox1* is more important in the oocyte for a successful fertilization.

Another possible explanation for the lack of progeny of *Hmox1*$^{-/-}$ could be an impaired implantation. In order to test this hypothesis, the embryos in two-cell stage obtained by *in vitro* fertilization were transferred into recipient females. To test whether *Hmox1* is necessary in the mother, in the fetus or in both for implantation to occur, *Hmox1*$^{-/-}$ oocytes were transferred into a *Hmox1*$^{+/+}$ or into a *Hmox1*$^{-/-}$ recipient female. Additionally, *Hmox1*$^{+/+}$ oocytes were transferred into a *Hmox1*$^{-/-}$ or into a *Hmox1*$^{+/+}$ female. The fact that *Hmox1*$^{-/-}$ oocytes were able to implant in a *Hmox1*$^{+/+}$ uterus confirms that *Hmox1*$^{-/-}$ oocytes do not present impaired hatching and can therefore implant in a uterus in which HO-1 is expressed. A work from Chen *et al.* shows that, after hatching, oocytes up-regulate HO-1 (Chen *et al.*, 2005). In the context of the experiments done for this thesis, the inability to up-regulate HO-1 in *Hmox1* deficient blastocysts may be compensated by the HO-1 expression in the maternal uterus. On the other hand, the fact that *Hmox1*$^{+/+}$ or *Hmox1*$^{-/-}$ oocytes were unable to implant in *Hmox1*$^{-/-}$ uteruses suggests that HO-1 is indispensable in the uterus for implantation to occur. Further, the sole expression of HO-1 by the blastocyst may not be sufficient to compensate the inflammatory milieu that the implantation of the embryo induces in the maternal uterus and cannot therefore contribute to the tolerance state needed after implantation for pregnancy to be maintained.

Having observed that HO-1 expression in the uterus is important or even indispensable for implantation to occur and that a lack of HO-1 leads to both, impaired implantation and impaired pregnancy, we next wondered whether HO-1 acts via T-cell dependent pathways. The results obtained when transferring *Hmox1*$^{+/+}$ or *Hmox1*$^{-/-}$ oocytes into SCID mice deficient in *Hmox1* are still very preliminary to make strong conclusions. By using SCID mice lacking T and B cells we next aimed to have a closer look on the participation of T cells in HO-1-dependent pregnancy success. If HO-1 mediated rejection operates via T cells, the lack of T

cells would then rescue from abortion. However, it was very interesting to observe that a higher percentage (around 13-15%) of *Hmox1*$^{-/-}$ progeny is obtained if the mice are in a SCID background (personal communication from Prof. Soares). In the experiments done for this work, *Hmox1*$^{+/+}$ oocytes were able to implant in a SCID female lacking HO-1. However, the SCID female used as a control did not implant, making the results difficult for interpretation. Nevertheless, the fact that more *Hmox1*$^{-/-}$ progeny is achieved in SCID background suggests that, in immune deficient animals, HO-1 may be not as necessary as in immune competent animals. This can be thought taking into account the immune cells present at the feto-maternal interface.

Summary and conclusion

1) Taken all these data together, it can be concluded that HO-1 is essential for successful pregnancy to occur. As it has been shown in this work, HO-1 is involved in different stages of pregnancy including:

2) Fertilization, as clearly shown by the significantly diminished fertilization rate obtained in *Hmox1*$^{-/-}$ combination when compared to a *Hmox1*$^{+/+}$ combination. This also suggests that HO-1 may be involved in ovulation and oocyte viability

3) Implantation, as shown by impairment of *Hmox1*$^{+/+}$ or *Hmox1*$^{-/-}$ oocytes in implanting in a *Hmox1*$^{-/-}$ but not in a *Hmox1*$^{+/+}$ female after embryo transfer.

4) Placentation, as shown *in vitro* by the inability of trophoblast stem cells to differentiate into giant cells in the absence of HO-1.

5) Avoidance of fetal rejection, as demonstrated *in vivo* by the diminution in the abortion rate in abortion-prone animals when injected with an adenoviral vector containing HO-1.

This data opens vast opportunities of further analyzing the role of this molecule in a more closely way in the different stages of pregnancy, and also helps to elucidate unknown mechanisms related to a process so important as the beginning of a new life.

References

Abbas AK, Lichtmann AH. Cellular and Molecular Immunology. Fifth ed. Philadelphia, USA: Saunders, Elsevier Science, 2003.

Abrahamsohn PA, Zorn TM. "Implantation and Decidualization in Rodents." J Exp Zool. 266, *no. 6* (1993): 603-28.

Adams KM, Nelson JL. Bi-Directional Cell Traficking During Pregnancy. Edited by Mor G, Immunology of Pregnancy. Georgetown, Texas, USA: Landes Bioscience/Eurekah.com, 2006.

Adin CA, Crocker BP, Agarwal A. "Protective effects of exogenous bilirubin on ischemia-reperfusion injury in the isolated, perfused rat kidney." Am J Physiol Renal Physiol. 288, *no. 4* (2005): F778-84.

Aft RL, Mueller GC. "Hemin-Mediated DNA Strand Scission." J Biol Chem. 258, *no. 19* (1983): 12069-72.

Aft RL, Mueller GC. "Hemin-Mediated Oxidative Degradation of Proteins." J Biol Chem. 259, *no. 1* (1984): 301-05.

Ahmad N. Identifizierung väterlicher fötaler Antigene in mütterlichen Organen während der Schwangerschaft anhand eines Mausmodells. Diploma thesis.

Akamatsu Y, Haga M, Tyagi S, Yamashita K, Graça-Souza AV, Ollinger R, Czismadia E, May GA, Ifedigbo E, Otterbein LE, Bach FH, Soares MP. "Heme Oxygenase-1-Derived Carbon Monoxide Protects Hearts from Transplant Associated Ischemia Reperfusion Injury." FASEB J. 18, *no. 6* (2004): 771-72.

Alberts B, Bray D, Hopkin K, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Essential Cell Biology. Second ed. New York, USA: Garland Science, Taylor & Francis Group, 2004.

Aluvihare VR, Kallikourdis M, Betz AG. "Regulatory T Cells Mediate Maternal Tolerance to the Fetus." Nat Immunol. 5, *no. 3* (2004): 266-71.

Amersi F, Buelow R, Kato H, Ke B, Coito AJ, Shen XD, Zhao D, Zaky J, Melinek J, Lassman CR, Kolls JK, Alam J, Ritter T, Volk HD, Farmer DG, Ghobrial RM, Busuttil RW, Kupiec-Weglinski JW. "Upregulation of Heme Oxygenase-1 Protects Genetically Fat Zucker Rat Livers from Ischemia/Reperfusion Injury." J Clin Invest 104, *no. 11* (1999): 1631-39.

Anderson RE, Williams WL. "Radiosensitivity of T and B Lymphocytes. V. Effects of Whole-Body Irradiation on Numbers of Recirculating T Cells and Sensitization to Primary Skin Grafts in Mice." Am J Pathol 89, *no. 2* (1977): 367-78.

Aoshiba K, Yokohori N, Nagai A. "Alveolar wall apoptosis causes lung destruction and emphysematous changes." Am J Respir Cell Mol Biol. 28 (2003): 555-62.

Aquaah G. Understanding Biotechnology. An Integrated and Cyber-based approach. Upper Saddle River, New Jersey, USA: Pearson Education, Inc., 2004.

Arck PC, Ferrick DA, Steele-Norwood D, Egan PJ, Croitoru K, Carding SR, Dietl J, Clark DA. "Murine T Cell Determination of Pregnancy Outcome." Cell Immunol. 196, *no. 2* (1999): 71-79.

Arvola M, Mattsson R. Placental Immune Defences., Encyclopedia of Life Sciences. Chichester, UK: John Wiley & Sons, Ltd, 2001.

Ashkar AA, Di Santo JP, Croy BA. "Interferon gamma contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy." Exp Med. 192, *no. 2* (2000):259-70.

Ashkar AA, Croy BA. "Functions of Uterine Natural Killer Cells Are Mediated by Interferon Gamma Production During Murine Pregnancy." Semin Immunol. 13, *no. 4* (2001): 235-41.

Attar E, Ozsait B, Bulgurcuoglu S, Serdaroglu H, Arici A. "Effect of Leukaemia Inhibitory Factor on Long-Term Sperm Motility and Survival." Reprod Biomed Online 7, *no. 1* (2003): 71-74.

Baban B, Chandler P, McCool D, Marshall B, Munn DH, Mellor AL. "Indoleamine 2,3-Dioxygenase Expression Is Restricted to Fetal Trophoblast Giant Cells During Murine Gestation and Is Maternal Genome Specific." J Reprod Immunol. 61, *no. 2* (2004): 67-77.

Bach FH, Hirschhorn K. "Lymphocyte Interaction: A Potential in Vitro Histocompatibility Test." Science, *no. 143* (1964): 813-14.

Bach FH. "Heme Oxygenase-1: A Therapeutic Amplification Funnel." FASEB J. 19, *no. 10* (2005): 1216-19.

Bach FH. "Carbon Monoxide: From the Origin of Life to Molecular Medicine." Trends Mol Med. 12, *no. 8* (2006): 348-50.

Bagley J, Bracy JL, Tian C, Kang ES, Iacomini J. "Establishing Immunological Tolerance through the Induction of Molecular Chimerism." Front Biosci 1, *no. 7* (2002): d1331-37.

Bain B, Vas MR, Lowenstein L. "The Development of Large Immature Mononuclear Cells in Mixed Leukocyte Cultures." Blood 23 (1964): 108-16.

Balla G, Vercellotti GM, Muller-Eberhard U, Eaton J, Jacob HS. "Exposure of endothelial cells to free heme potentiates damage mediated by granulocytes and toxic oxygen species." Lab Invest. 64, *no. 5* (1991):648-55.

Balla G, Jacob HS, Balla J, Rosenberg M, Nath K, Apple F, Eaton JW, Vercellotti GM. "Ferritin: A Cytoprotective Antioxidant Strategem of Endothelium." J Biol Chem 267, *no. 25* (1992): 18148-53.

Balla J, Balla G, Jeney V, Kakuk G, Jacob HS, Vercellotti GM. "Ferriporphyrins and Endothelium: A 2-Edged Sword-Promotion of Oxidation and Induction of Cytoprotectants." Blood 95, *no. 11* (2000): 3442-50.

Bangari DS, Mittal SK. "Current strategies and future directions for eluding adenoviral vector immunity." Curr Gene Ther. 6, *no. 2* (2000): 215-26.

Barber A, Robson SC, Myatt L, Bulmer J, Lyall F. "Heme Oxygenase Expression in Human Placenta and Placental Bed: Reduced Expression of Placenta Endothelial Ho-2 in Pre-Eclampsia and Fetal Growth Restriction." FASEB J 15 (2001): 1158-68.

Benirschke K, Kaufmann P. Placental Types. Fourth ed, Pathology of Human Placenta. New York, USA: Springer, 2001.

Berberat PO, Katori M, Kaczmarek E, Anselmo D, Lassman C, Ke B, Shen X, Bussutil RW, Yamashita K, Csizmadia E, Tyaqi S, Otterbein LE, Brouard S, Tobiasch E, Bach FH, Kupiec-Weglinski JW, Soares MP. "Heavy chain ferritin acts as an antipoptotic gene that protects livers from ischemia reperfusion injury." FASEB J. 17*, no. 12* (2003): 1724-6.

Bergh PA, Navot D. "The Impact of Embryonic Development and Endometrial Maturity on the Timing of Implantation." Fertil Steril. 58, *no. 3* (1992): 537-42.

Bertoja AZ, Zenclussen ML, Wollenberg I, Paeschke S, Sollwedel A, Gerlof K, Woicie-chowsky C, Volk HD, Zenclussen AC. "Upregulation of Bcl-2 at the Foetal-Maternal Interface from Mice Undergoing Abortion." Scand J Immunol. 61, *no. 6* (2005): 492-502.

Bianchi, DW, Zickwolf GK, Weil GJ, Sylvester S, DeMaria MA. "Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum." Proc Natl Acad Sci USA 93, *no. 2* (1996): 705-8.

Bilban M, Bach FH, Otterbein SL, Ifedigbo E, de Costa d'Avila J, Esterbauer H, Chin BY, Usheva A, Robson SC, Wagner O, Otterbein LE. "Carbon Monoxide Orchestrates a Protective Response through Ppargamma." Immunity. 24, *no. 5* (2006): 601-10.

Blaese RM, Culver KW, Miller AD, Carter CS, Fleisher T, Clerici M, Shearer G, Chang L, Chiang Y, Tolstoshev P, Greenblatt JJ, Rosenberg SA, Klein H, Berger M, Mullen CA, Ramsey WJ, Muul L, Morgan RA, Anderson WF. "T Lymphocyte-Directed Gene Therapy for Ada- Scid: Initial Trial Results after 4 Years." Science 270*, no. 5235* (1995): 475-80.

Boulikas T. "Status of Gene Therapy in 1997: Molecular Mechanisms, Disease Targets, and Clinical Applications." Gene Ther Mol Biol 1 (1998): 1-172.

Braudeau C, Bouchet D, Tesson L, Iyer S, Rémy S, Buelow R, Anegon I, Chauveau C. "Induction of long-term cardiac allograft survival by heme oxygenase-1 gene transfer." Gene Ther. 11, *no. 8* (2004): 701-10.

Brouard S, Otterbein LE, Anrather J, Tobiasch E, Bach FH, Choi AM, Soares MP. "Carbon Monoxide Generated by Heme Oxygenase 1 Suppresses Endothelial Cell Apoptosis." J Exp Med. 192, *no. 7* (2000): 1015-26.

Bruder D, Probst-Kepper M, Westendorf AM, Geffers R, Beissert S, Loser K, von Boehmer H, Buer J, Hansen W. "Neuropilin-1: A Surface Marker of Regulatory T Cells." Eur J Immunol 34, *no. 3* (2004): 623-30.

Buchschader GL, Wong-Stall F. "Development of lentiviral vectors for gene therapy for human diseases." Blood 95 (2000): 2499-504.

Buelow R, Tullius SG, Volk HD. "Protection of Grafts by Hemoxygenase-1 and Its Toxic Product Carbon Monoxide." Am J Transplant. 1, *no. 4* (2001): 313-15.

Burns JC, Friedmann T, Driever T, Burrascano M, Yee J-K. "Vesicula Stomatitis Virus G Glycoprotein Pseudotyped Retroviral Vectors: Concentration to Very High Titer and Efficient Gene Transfer into Mammalian and Non Mammalian Cells." Proc Natl Acad Sci USA 90, *no. 17* (1993): 8033-37.

Bussolati B, Ahmed A, Pemberton H, Landis RC, Di Carlo F, Haskard DO, Mason JC. "Bifunctional role for VEGF-induced heme oxygenase-1 in vivo: induction of angiogenesis and inhibition of leukocytic infiltration." Blood 103, *no. 3* (2004): 761-6.

Chai JG, Tsang JY, Lechler R, Simpson E, Dyson J, Scott D. "Cd4+Cd25+ T Cells as Immunoregulatory T Cells in Vitro." Eur J Immunol 32*, no. 8* (2002): 2365-75.

Chaouat G, Clark DA, Wegmann TG. Genetic Aspects of the Cba X Dba/2 and B10 X B10.A Models of Murine Pregnancy Failure and Its Prevention by Lymphocyte Immunisation. Edited by Sharp F Beard RW, Early Pregnancy Loss: Mechanisms and Treatment. London, UK: Springer, 1988.

Chaouat, G., Meliani AA, Martal J, Raghupathy R, Elliot J, Mosmann T, Wegmann T. "Il-10 Prevents Naturally Occurring Fetal Loss in the Cba X Dba/2 Mating Combination, and Local Defect in Il-10 Production in This Abortion- Prone Combination Is Corrected by in Vivo Injection of Ifn-Tau." J. Immunol 154 (1995): 4261-68.

Chapel H, Haeney M, Misbah S, Snowden N. Essentials of Clinical Immunology. Fourth ed. London, UK: Blackwell Science, 1999.

Chauveau C, Bouchet D, Roussel JC, Mathieu P, Braudeau C, Renaudin K, Tesson L, Soulillou JP, Iyer S, Buelow R, Anegon I. "Gene transfer of heme oxygenase-1 and carbon monoxide delivery inhibit chronic rejection." Am J Transpl. 2, *no. 7* (2002): 581-92.

Chauveau C, Rémy S, Royer PJ, Hill M, Tanguy-Royer S, Hubert FX, Tesson L, Brion R, Beriou G, Gregoire M, Josien R, Cuturi MC, Anegon I. "Heme oxygenase-1 expression inhibits dendritic cell maturation and pro-inflammatory function but conserves IL-10 expression." Blood 106, *no. 5* (2005): 1694-702.

Chen HW, Chen JJ, Yu SL, Li HN, Yang PC, Su CM, Au HK, Chang CW, Chien LW, Chen CS, Tzeng CR. "Transcriptome analysis in blastocyst hatching by cDNA microarray." Hum Reprod. 20, *no. 9* (2005): 2492-501.

Choi BM, Pae HO, Jeong YR, Kim YM, Chung HT. "Critical role of heme oxygenase-1 in Foxp3-mediated immune suppression." Biochem Biophys Res Commun. 327, *no. 4* (2005): 1066-71.

Chora AA, Fontoura P, Cunha A, Pais TF, Cardoso S, Ho PP, Lee LY, Sobel RA, Steinman L, Soares MP. "Heme Oxygenase-1 and carbon monoxide suppress autoimmune neuroinflammation." J Clin Invest. 117, *no. 2* (2007): 438-47.

Claas FH. "Predictive Parameters for in Vivo Alloreactivity." Transpl Immunol 10, *no. 2-3* (2002): 137-42.

Clark DA, McDermott MR, Szewczuk MR. "Impairment of Host-Versus-Graft Reaction in Pregnant Mice: Ii) Selective Suppression of Cytotoxic Cell Generation Correlates with Soluble Suppressor Activity and with Successful Allogeneic Pregnancy." Cell Immunol. 52, *no. 1* (1980): 106-18.

Clark DA, Merali FS, Hoskin DW, Steel-Norwood D, Arck PC, Croitoru K, Murgita RA, Hirte H. "Decidua-Associated Suppressor Cells in Abortion-Prone Dba/2-Mated Cba/J Mice That Release Bioactive Transforming Growth Factor Beta2-Related Immunosuppressive Molecules Express a Bone Marrow-Derived Natural Suppressor Cell Marker and Gamma Delta T-Cell Receptor." Biol Reprod 56 (1997): 1351-60.

Coburn RF, Williams WJ, White P, Kahn SB. "The Production of Carbon Monoxide from Hemoglobin in Vivo." J Clin Invest. 46, *no. 3* (1967): 346-56.

Converso DP, Taillé C, Carreras MC, Jaitovich A, Poderoso JJ, Boczkowski J. "HO-1 is located in liver mitochondria heme content and metabolism." FASEB J. 20, *no.8* (2006): 1236-8

Coito AJ, Buelow R, Shen XD, Amersi F, Moore C, Volk HD, Busuttil RW, Kupiec-Weglinski JW. "Heme Oxygenase-1 Gene Transfer Inhibits Inducible Nitric Oxide Synthase Expression and Protects Genetically Fat Zucker Rat Livers from Ischemia-Reperfusion Injury." Transplantation 74, *no. 1* (2002): 96-102.

Christiansen OB, Nielsen HS, Kolte AM. "Inflammation and miscarriage." Semin Fetal Neonatal Med. 11 (2006): 302-8.

Cross JC. "How to make a placenta: mechanisms of trophoblast cell differentiation in mice- a review." Placenta 26 Supplement A, Trophoblast Research 19 (2005): S3-9.

Croy BA, McBey BA, Villeneuve LA, Kusakabe K, Kiso Y, van den Heuvel M. "Characterization of the Cells That Migrate from Metrial Glands of the Pregnant Mouse Uterus During Explant Culture." J Reprod Immunol. 32, *no. 3* (1997): 241-63.

Dai Y, Schwarz EM, Gu D, Zhang WW, Sarvetnick N, Verma IM. "Cellular and humoral immune responses to adenoviral vectors containing factor IX gene: tolerization of factor IX and vector antigens allows for long-term expression." Proc Natl Acad Sci USA 92 (1995): 1401-5.

Danos O, Mulligan RC. "Safe and Efficient Generation of Recombinant Retroviruses with Amphotropic and Ecotropic Host Ranges." Proc Natl Acad Sci U S A 85, *no. 17* (1988): 6460-64.

Drazen DL, Klein SL, Burnett AL, Wallach EE, Crone JK, Huang PL, Nelson RJ. "Reproductive function in female mice lacking the gene for endothelial nitric oxide synthase." Nitric Oxide 3 (1999): 366-74.

Dulak J, Motterlini R, Huk I, Pachinger O, Weidinger F, Józkowicz A. Carbon monoxide and iron, by-products of heme oxygenase, modulate vascular endothelial growth factor synthesis in vascular smooth muscle cells. Edited by NG Abraham, Alam J, Nath K. Heme Oxygenase in Biology and Medicine. New York, USA: Kluwer Academic/Plenum Publishers, 2002.

Dulak J, Loboda A, Zagorska A, Jozkowicz A. "Complex role of heme oxygenase-1 in angiogenesis." Antioxid redox Signal. 6, *no. 5* (2004): 858-66.

Ejima K, Perrella MA. "Alteration in heme oxygenase-1 and nitric oxide synthase-2 gene expression during endotoxemia in cyclooxygenase-2 deficient mice." Antioxid. Redox Signal. 6, *no. 5* (2004): 850-7.

Elbe-Bürger A, Mommaas AM, Prieschl EE, Fiebiger E, Baumruker T, Stingl G. "Major histocompatibility complex class II- fetal skin dendritic cells are potent accesory cells of polyclonal T-cell responses." Immunology 101, *no. 2* (2000): 242-53.

Espey LL, Bellinger AS, Healy JA. Ovulation: An Inflammatory Cascade of Gene Expression, The Ovary, 2004.

Everett RS, Evans HK, Hodges BL, Ding EY, Serra DM, Amalfitano A. "Strain-specific rate of shutdown of CMV enhancer activity in murine liver confirmed by use of persistent [E1-, E2b-] adenoviral vectors." Virology 325 (2004): 96-105.

Fallaux, F. J., O. Kranenburg, S. J. Cramer, A. Houweling, H. Van Ormondt, R. C. Hoeben, A. J. Van Der Eb. "Characterization of 911: A New Helper Cell Line for the Titration and Propagation of Early Region 1-Deleted Adenoviral Vectors." Hum Gene Ther 7 (1996): 215-22.

Fallon PG, Jolin HE, Smith P, Emson CL, Townsed MJ, Fallon R, Smith P, McKenzie AN. "IL-4 induces characteristic Th2 even in the combined absence of IL-5, IL-19 and IL13." Immunity 17 (2002): 7-17.

Faria TN, Soares MJ. "Trophoblast Cell Differentiation: Establishment, Characterization, and Modulation of Rat Trophoblast Cell Line Expressing Members of the Placental Prolactin Family." Endocrinology 129, *no. 6* (1991): 2895-906.

Finn CA, Martin L. "Patterns of Cell Division in the Mouse Uterus During Early Pregnancy." J Endocrinol. 39, *no. 4* (1967): 593-97.

Fujii H, Takahashi T, Nakahira K, Uehara K, Shimizu H, Matsumi M, Morita K, Hirakawa M, Akaqi R, Sassa S. "Protective role of heme oxygenase-1 in the intestinal tissue injury in an experimental model of sepsis." Crit Care Med. 31, *no. 3* (2003): 893-902.

Fujita T, Toda K, Karimova A, Yan S-F, Naka Y, Yet S-F, Pinsky DJ. "Paradoxical rescue from ischemic lung injury by inhaled carbon monoxide driven by derepression of fibronolysis." Nat Med. 7, *no. 5* (2001): 598-604.

Glasgow JN, Everts M, Curiel DT. "Transductional targeting of adenovirus vectors for gene therapy." Cancer Gene Ther. 13, *no. 9* (2006): 830-44.

Gobé G, Harmon B. Apoptosis*:* M Orphological Criteria and Other Assays, Encyclopedia of Life Sciences. Chichester, UK: John Wiley & Sons Ltd, 2005.

Graham, F., Prevec L. Gene Transfer and Expression Protocols. Edited by Clifton NJ Murray EJ, Methods in Molecular Biology: The Humana Press Inc., 1991.

Graham FL. "Adenoviruses vectors for high-efficiency gene transfer into mammalian cells." Immunol Today 21 (2000): 426-8.

Granick S, Sinclair P, Sassa S, Grieninger G. "Effects by Heme, Insulin, and Serum Albumin on Heme and Protein Synthesis in Chick Embryo Liver Cells Cultured in a Chemically Defined Medium, and a Spectrofluorometric Assay for Porphyrin Composition." J Biol Chem. 250, *no. 24* (1975): 9215-25.

Gregori S, Casorati M, Amuchastegui S, Smiroldo S, Davalli AM, Adorini L. "Regulatory T Cells Induced by 1 Alpha,25-Dihydroxyvitamin D3 and Mycophenolate Mofetil Treatment Mediate Transplantation Tolerance." J Immunol. 167, *no. 4* (2001): 1945-53.

Hambartsoumian E. "Endometrial Leukaemia Inhibitory Factor (Lif) as a Possible Cause of Unexplained Infertility and Multiple Failures of Implantation." Am J Reprod Immunol. 39, *no. 2* (1998): 137-43.

Hammer MH, Flugel A, Seifert M, Lehmann M, Brandt C, Volk HD, Ritter T. "Potential of Allospecific Gene-Engineered T Cells in Transplantation Gene Therapy: Specific T Cell Activation Determines Transgene Expression in Vitro and in Vivo." Hum Gene Ther. 11, *no. 9* (2000): 1303-11.

Hammer MH, Schroder G, Risch K, Flugel A, Volk HD, Lehmann M, Ritter T. "Antigen-Dependent Transgene Expression in Kidney Transplantation: A Novel Approach Using Gene-Engineered T Lymphocytes." J Am Soc Nephrol. 13, *no. 2* (2002): 511-18.

Hara M, Kingsley CI, Niimi M, Read S, Turvey SE, Bushell AR, Morris PJ, Powrie F, Wood KJ. "Il-10 Is Required for Regulatory T Cells to Mediate Tolerance to Alloantigens in Vivo." J Immunol. 166, *no. 6* (2001): 3789-96.

Hefler LA, Gregg AR. "Inducible and endothelial nitric oxide synthase: genetic background affects ovulation in mice." Fertil Steril 77 (2002): 147-51.

Hegazi RA, Rao KN, Mayle A, Sepulveda AR, Otterbein LE, Plevy SE. "Carbon Monoxide Ameliorates Chronic Murine Colitis through a Heme Oxygenase 1-Dependent Pathway." J Exp Med. 202, *no. 12* (2005): 1703-13.

Heikkinen J, Möttönen M, Alanen A, Lassila O. "Phenotypic Characterization of Regulatory T Cells in the Human Decidua." Clin Exp Immunol. 136, *no. 2* (2004): 373-78.

Henderson AR. "Testing experimental data for univariate normality." Clinica Chimica Acta 366 (2006): 112-29.

Hilton DJ, Gough NM. "Leukemia Inhibitory Factor: A Biological Perspective." J Cell Biochem. 46*, no. 1* (1991): 21-26.

Hori S, Nomura T, Sakaguchi S. "Control of regulatory T cell development by the transcription factor FoxP3." Science 229 (2003): 1057-61.

Huang, Z. "Kinetic fluorescence measurement of fluorescein di-□-D-galactosidase hydrolysis by □-galactosidase: intermediate channeling in stepwise catalysis by a free single enzyme." Biochemistry 30 (1991): 8535-40.

Huard J, Lochmüller H, Acsadi G, Jani A, Massie B, Karpati G. "The Route of Administration Is a Major Determinant of the Transduction Efficiency of Rat Tissues by Adenoviral Recombinants." Gene Ther. 2, *no. 2* (1995): 107-15.

Hunt JS. "Cytokine Networks in the Uteroplacental Unit: Macrophages as Pivotal Regulatory Cells." J Reprod Immunol. 16, *no. 1* (1989): 1-17.

Hunt JS, Miller L, Vassmer D, Croy BA. "Expression of the Inducible Nitric Oxide Synthase Gene in Mouse Uterine Leukocytes and Potential Relationships with Uterine Function During Pregnancy." Biol Reprod. 57, *no. 4* (1997): 827-36.

Hunt JS, Petroff MG, Burnett TG. "Uterine Leukocytes: Key Players in Pregnancy." Semin Cell Dev Biol. 11, *no. 2* (2000): 127-37.

Hunt JS, Petroff MG, McIntire RH, Ober C. "Hla-G and Immune Tolerance in Pregnancy." FASEB J. 19, *no. 7* (2005): 681-93.

Ihara N, Akagi R, Ejiri K, Kudo T, Furuyama K, Fujita H. "Developmental changes of gene expression in heme metabolic enzymes in rat placenta." FEBS Lett. 439 (1998): 163–167.

Jablonka-Shariff A, Olson LM. "The role of nitric oxide in oocyte meiotic maturation and ovulation: meiotic abnormalities of endothelial nitric oxide synthase knock-out mouse oocytes." Endocrinology 139 (1998): 2944-54.

Jerzak M, Bischof P. "Apoptosis in the First Trimester Human Placenta: The Role in Maintaining Immune Privilege at the Maternal-Foetal Interface and in the Trophoblast Remodelling." Eur J Obstet Gynecol Reprod Biol. 100, *no. 2* (2002): 138-42.

Johnson RA, Kozma F, Colombari E. "Carbon monoxide: from toxin to endogenous modulator of cardiovascular functions." Braz. J. Med. Biol. Res. 32 (1999): 1–14.

Juan SH, Lee TS, Tseng KW, Liou JY, Shyue SK, Wu KK, Chau LY. "Adenovirus-mediated heme oxygenase-1 gene transfer inhibits the development of atherosclerosis in apolipoprotein E-deficient mice." Circulation 104, *no. 13* (2001): 1519-25.

Juretic K, Strbo N, Crncic TB, Laskarin G, Rukavina D. "An Insight into the Dendritic Cells at the Maternal-Fetal Interface." Am J Reprod Immunol. 52, *no. 6* (2004): 350-55.

Kamimura S, Eguchi K, Yonezawa M, Sekiba K. "Localization and Developmental Change of Indoleamine 2,3-Dioxygenase Activity in the Human Placenta." Acta Med Okayama. 45, *no. 3* (1991): 135-39.

Katori M, Busuttil RW, Kupiec-Weglinski JW. "Heme Oxygenase-1 System in Organ Transplantation." Transplantation. 74, *no. 7* (2002): 905-12.

Kay MA, Landen CN, Rothenberg SR, Taylor LA, Leland F, Wiehle S, Fang B, Bellinger D, Finegold M, Thompson AR, Read M, Brinkhous KM, Woo SLC**.** "In vivo hepatic gene therapy: complete albeit transient correction of factor IX deficiency in hemophilia B dogs." Proc. Natl. Acad. Sci. USA. 91*, no. 6* (1994): 2353-7.

Kazuo S, Kenichi T, Toshiaki Y, Yasuo T, Toshinobu M, Mutsuo I. "Effects of low concentrations of nitric oxide on the zona pellucida binding ability of human spermatozoa." Fertil Steril 69 (1998): 522-7.

Kim HP, Wang X, Galbiati F, Ryter SW, Choi AM. "Caveolae compartmentalization of heme oxygenase-1 in endothelial cells." FASEB J. 18, *no. 10* (2004): 1080-9.

Kingsley CI, Karim M, Bushell AR, Wood KJ. "Cd25+Cd4+ Regulatory T Cells Prevent Graft Rejection: Ctla-4- and Il-10-Dependent Immunoregulation of Alloresponses." J Immunol. 168, *no. 3* (2002): 1080-86.

Koi H, Zhang J, Makrigiannakis A, Getsios S, MacCalman CD, Kopf GS, Straus JF 3rd, Parry S. "Differential Expression of the Coxsackievirus and Adenovirus Receptor Regulates Adenovirus Infection of the Placenta." Biol Reprod. 64, *no. 3* (2001): 1001-09.

Koizumi S. "Human heme oxygenase-1 deficiency: a lesson on serendipity in the discovery of a novel disease." Pediatr Int. 49, *no. 2* (2007): 125-32.

Khosrotehrani K, Johnson KL, Guégan S, Stroh H, Bianchi S. "Natural history of fetal cell microchimerism during and following murine pregnancy." J Reprod Immunol. 66, *no. 1* (2005): 1-12.

Kozarsky KF, Jooss K, Donahee M, Strauss JF 3rd, Wilson JM. "Effective treatment of familial hypercholesterolaemia in the mouse model using adenovirus-mediated transfer of the VLDL receptor gene". Nat Genet. 13, *no. 3* (1996): 374.

Kreiser D, Nguyen X, Wong R, Seidman D, Stevenson D, Quan S, Abraham N, Dennery PA. "Heme Oxygenase-1 Modulates Fetal Growth in the Rat." Lab Invest. 82 (2002): 687-92.

Krishnan, L., L. J. Guilbert, T. G. Wegmann, M. Belosevic, T. R. Mossmann. "T Helper 1 Response against Leishmania Major in Pregnant C57bl/6 Mice Increases Implantation Failure and Fetal Resorptions. Correlation with Increased Ifn-Gamma and Tnf-Alpha and Reduced Il-10 Production by Placenta Cells." J Immunol. 15 (1996): 653-62.

Lang D, Reuter S, Buzescu T, August C, Heidenreich S. "Heme-induced heme oxygenase-1 (HO-1) in human monocytes inhibits apoptosis despite caspase-3 up-regulation." Int Immunol. 17*, no. 2* (2005):155-65.

Laurema A, Vanamo K, Heikkilä A, Riekkinen M, Heinonen S, Ylä-Herttuala S. "Fetal Membranes Act as a Barrier for Adenoviruses: Gene Transfer into Exocoelomic Cavity of Rat Fetuses Does Not Affect Cells in the Fetus." Am J Obstet Gynecol. 190, *no. 1* (2004): 264-67.

Lea RG, Riley SC, Antipatis C, Hannah L, Ashworth CJ, Clark DA, Critchley HO. "Cytokines and the Regulation of Apoptosis in Reproductive Tissues: A Review." Am J Reprod Immunol. 42, *no. 2* (1999): 100-09.

Lee SS, Gao W, Mazzola S, Thomas MN, Csizmadia E, Otterbein LE, Bach FH, Wang H. "Heme oxygenase-1, carbon monoxide, and bilirubin induce tolerance in recipients toward islet allografts by modulating T regulatory cells." FASEB J. 21, *no. 13* (2007): 3450-7.

Liang S, Horuzsko A. "Mobilizing Dendritic Cells for Tolerance by Engagement of Immune Inhibitory Receptors for Hla-G." Hum Immunol. 64, *no. 11* (2003): 1025-32.

Lim H, Paria BC, Das SK, Dinchuk JE, Langenbach R, Trzaskos JM, Dey SK. "Multiple female reproductive failures in cyclooxygenase 2-deficient mice." Cell 91 (1997): 197-208.

Lin F, Girotti AW. "Hemin-Enhanced Resistance of Human Leukemia Cells to Oxidative Killing: Antisense Determination of Ferritin Involvement." Arch Biochem Biophys. 352, *no. 1* (1998): 51-58.

Lin H, Everse J. "The Cytotoxic Activity of Hematoheme: Evidence for Two Different Mechanisms." Anal Biochem. 161, *no. 2* (1987): 323-31.

Lin H, Mossmann TR, Guilbert L, Tuntipopipat S, Wegmann TG. "Synthesis of T Helper 2-Type Cytokines at the Feto-Maternal Interface." J Immunol. 151 (1993): 4562-73.

Lin Q, Wels S, Yang G, Weng Y-H, Helston R, Rish K, Smith A, Bordner J, Polte T, Gaunitz F, Dennery PA. "Heme Oxygenase-1 protein localizes to the nucleus and activates transcription factors important in oxidative stress." J Biol Chem. 282, *no. 28* (2007): 20621-33.

Linnemeyer PA, Pollack SB. "Stage-Specific Expression of Activation Antigens on Nk Cells at Uterine Implantation Sites in Mice." J Immunol. 153, *no. 4* (1994): 1478-86.

Lo YM, Lo ES, Watson N, Noakes L, Sargent IL, Thilaganathan B, Wainscoat JS. "Two-way cell traffic between mother and fetus: biologic and clinical implications." Blood 88, *no. 11* (1996): 4390-5.

Luttmann W, Bratke K, Küpper M, Myrtek D. Der Experimentator- Immunologie. First ed., Munich, Germany, Spektrum Akademischer Verlag, Elsevier GmbH, 2004.

Lyall F, Barber A, Myatt L, Bulmer J, Robson SC. "Hemeoxygenase expression in human placenta and placental bed implies a role in regulation of trophoblast invasion and placental function." FASEB J. 14 (2000): 208-19.

MacCauley A, Cross JC, Werb Z. "Reprogramming the cell cycle for endoreduplication in rodent trophoblast cells." Mol Biol Cell 9 (1998): 795-807.

MacCalman CD, Furth EE, Omigbodun A, Kozarsky KF, Coutifaris C, Strauss JF. "Transduction of Human Trophoblast Cells by Recombinant Adenoviruses Is Differentiation Dependent." Biol Reprod. 54, *no. 3* (1996): 682-91.

Maines MD, Kappas A. "Cobalt Induction of Hepatic Heme Oxygenase; with Evidence That Cytochrome P-450 Is Not Essential for This Enzyme Activity." Proc Natl Acad Sci U S A. 71, *no. 11* (1974): 4293-97.

Maines MD, Kappas A. "Cobalt Stimulation of Heme Degradation in the Liver. Dissociation of Microsomal Oxidation of Heme from Cytochrome P-450." J Biol Chem. 250, *no. 11* (1975): 4171-77.

Maines MD. "New Developments in the Regulation of Heme Metabolism and Their Implications." Crit Rev Toxicol. 12, *no. 3* (1984): 241-314.

Maines MD, Trakshel GM, Kutty RK. "Characterization of Two Constitutive Forms of Rat Liver Microsomal Heme Oxygenase. Only One Molecular Species of the Enzyme Is Inducible." J Biol Chem. 261, *no. 1* (1986): 411-19.

Maines MD. Heme Oxygenase and Heme Degrading Systems, Heme Oxygenase: Clinical Implications and Functions. Boca Raton, USA: CRC Press, 1992.

Maines MD, Eke BC, Zhao X. "Corticosterone Promotes Increased Heme Oxygenase-2 Protein and Transcript Expression in the Newborn Rat Brain." Brain Res. 722, *no. 1-2* (1996): 83-94.

Maines MD. "The Heme Oxygenase System: A Regulator of Second Messenger Gases." Annu Rev Pharmacol Toxicol. 37 (1997): 517-54.

Maines MD, Panahian N. The Heme Oxygenase System and Cellular Defense Mechanisms. Edited by Roach RC, Hypoxia: From Genes to the Bedside. New York, USA: Kluwer Academic/Plenum Publishers, 2001.

Malan Borel I, Gentile T, Angelucci J, Pividori J, Guala MC, Binaghi RA, Margni RA. "Igg Asymmetric Molecules with Antipaternal Activity Isolated from Sera and Placenta of Pregnant Human." J Reprod Immunol. 20, *no. 2* (1991): 129-40.

Malinowski K, Pullis C, Raisbeck AP, Rapaport FT. "Modulation of Human Lymphocyte Marker Expression by Gamma Irradiation and Mitomycin C." Cell Immunol. 143, *no. 2* (1992): 368-77.

Maloney S, Smith A, Furst DE, Myerson D, Rupert K, Evans PC, Nelson JL. "Microchimerism of maternal origin persists into adult life." J Clin Invest. 104, *no. 1* (1999): 41-7.

Margni RA. "Coprecipitating Igg Asymmetric Antibodies: A Possible Role for Fab Glycosylation, and Speculations on Their Formation and Functions in Disease." Glycosilation and Disease. (1994): 59-66.

Margni RA, Binaghi RA. "Nonprecipitating Asymmetric Antibodies." Annu Rev Immunol. 6 (1988): 535-54.

Markowitz D, Goff S, Bank A. "A Safe Packaging Line for Gene Transfer: Separating Viral Genes on Two Different Plasmids." J Virol. 62, *no. 4* (1988): 1120-24.

Mathieu P, Chauveau C, Bouchet D, Guillot C, Tesson L, Anegon I. "Genetic Engineering in Allotransplantation of Vascularized Organs." Curr Gene Ther. 2, *no. 1* (2002): 9-21.

Matsumoto H, Ma W, Smalley W. Trzaskos J, Breyer RM, Dey SK. "Diversification of cyclooxygense-2-derived prostaglandins in ovulation and implantation." Biol Reprod. 64 (2001): 1557-65.

McCoubrey WK, Huang TJ, Maines MD. "Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3." Eur J Biochem. 247 (1997): 725-32.

McGarry HF, Plant LD, Taylor MJ. "Diethylcarbamazine activity against Brugia malayi microfilariae is dependent on inducible nitric-oxide synthase and the cyclooxygenase pathway." Filaria J. 4 (2005): 4.

Medawar PB. Some Immunological and Endcrinological Problems Raised by Evolution of Viviparity in Vertebrates. Edited by Symposia of the Society for Experimental Biology. Vol. Number 7, Evolution. London, UK: Syndics of the Cambridge University Press, 1953.

Mellor AL, Munn DH. "Tryptophan Catabolism and T-Cell Tolerance: Immunosuppression by Starvation?" Immunol Today. 20, *no. 10* (1999): 469-73.

Montellano PR. "The Mechanism of Heme Oxygenase." Curr Opin Chem Biol. 4, *no. 2* (2000): 221-27.

Mor G, Abrahams VM. "Potential Role of Macrophages as Immunoregulators of Pregnancy." Reprod Biol Endocrinol. 1 (2003): 119.

Morris MC, Depollier J, Mery J, Heitz F, Divita G. "A peptide carrier for the delivery of biologically active proteins into mammalian cells." Nature Biotechnology 19 (2001): 1173-6.

Morse D, Choi AM. "Heme Oxygenase-1: The "Emerging Molecule" Has Arrived." Am J Respir Cell Mol Biol. 27, *no. 1* (2002): 8-16.

Munn DH, Zhou M, Attwood JT, Bondarev I, Conway SJ, Marshall B, Brown C, Mellor AL. "Prevention of Allogeneic Fetal Rejection by Tryptophan Catabolism." Science 281, *no. 5380* (1998): 1191-93.

Nagy A, Gertsenstein M, Vintersten K, Behringer R. Manipulating the Mouse Embryo: A Laboratory Manual. New York, USA: Cold Spring Harbor Laboratory Press, 2003.

Nakao A, Neto JS, Kanno S, Stolz DB, Kimikuza K, Liu F, Bach FH, Billiar TR, Choi AM, Otterbein LE, Murase N. "Protection against ischemia/reperfusion injury in cardiac and renal transplantation with carbon monoxide, biliverdin and both." Am J Transplant. 5, *no. 2* (2005): 282-91.

Nath KA, Balla G, Vercellotti GM, Balla J, Jacob HS, Levitt MD, Rosenberg ME. "Induction of Heme Oxygenase Is a Rapid, Protective Response in Rhabdomyolysis in the Rat." J Clin Invest. 90, *no. 1* (1992): 267-70.

Nathan C. "Nitric Oxide as a Secretory Product of Mammalian Cells." FASEB J. 6, *no. 12* (1992): 3051-64.

Neuzil J, Stocker R. "Bilirubin Attenuates Radical-Mediated Damage to Serum Albumin." FEBS Lett. 331, *no. 3* (1993): 281-84.

Nolan GP, Fiering S, Nicolas JF, Herzenberg LA. "Fluorescence-activated cell analysis and sorting of viable mammalian cells based on □-D-galactosidase activity after transduction of Escherichia coli lacZ." Proc. Natl. Acad. Sci. USA 85 (1988): 2603-7.

North AJ, Lau KS, Brannon TS, Wu LC, Wells LB, German Z, Shaul PW. "Oxygen Upregulates Nitric Oxide Synthase Gene Expression in Ovine Fetal Pulmonary Artery Endothelial Cells." Am J Physiol. 270*, no. 4 Pt 1* (1996): L643-49.

Okada Y, Ueshin Y, Isotani A, Saito-Fujita T, Nakashima H, Kimura K, Mizoguchi A, Oh-hora M, Mori Y, Ogata M, Oshima RG, Okabe M, Ikawa M. "Complementation of placental defects and embryonic lethality of trophoblast specific lentiviral gene transfer." Nat Biotechnol. 25, *no. 2* (2007): 233-7.

Otterbein LE, Bach FH, Alam J, Soares M, Tao Lu H, Wysk M, Davis RJ, Flavell RA, Choi AM. "Carbon Monoxide Has Anti-Inflammatory Effects Involving the Mitogen-Activated Protein Kinase Pathway." Nat Med. 6, *no. 4* (2000): 422-28.

Otterbein LE, Choi AM. "Heme Oxygenase: Colors of Defense against Cellular Stress." Am J Physiol Lung Cell Mol Physiol 279*, no. 6* (2000): L1029-37.

Pae HO, Oh GS, Choi BM, Chae SC, Chung HT. "Differential Expressions of Heme Oxygenase-1 Gene in Cd25- and Cd25+ Subsets of Human Cd4+ T Cells." Biochem Biophys Res Commun. 306, *no. 3* (2003): 701-05.

Pae HO, Oh GS, Choi BM, Chae SC, Kim YM, Chung KR, Chung HT. "Carbon Monoxide Produced by Heme Oxygenase-1 Suppresses T Cell Proliferation Via Inhibition of Il-2 Production." J Immunol. 172, *no. 8* (2004): 4744-51.

Pala P, Hussell T, Openshaw PJM. "Flow cytometric measurement of intracellular cytokines." J Immunol Methods 243 (2000): 107-24.

Pamplona A, Ferreira A, Balla J, Jeney V, Balla G, Epiphano S, Chora A, Rodrigues CD, Gregoire IP, Cunha-Rodrigues M, Portugal S, Soares MP, Mota MM. "Heme oxygenase-1 and carbon monoxide suppress the pathogenesis of experimental cerebral malaria." Nat Med. 13, *no. 6* (2007): 703-10.

Parry S, Holder J, Halterman MW, Weitzman MD, Davis AR, Federoff H, Strauss JF 3rd. "Transduction of human trophoblastic cells by replication-deficient recombinant viral vectors. Promoting cellular differentiation affects virus entry." Am J Pathol. 152, *no. 6* (1998):1521-9.

Peters TJ, Chapman BM, Soares MJ. Trophoblast Differentiation: An in Vitro Model for Trophoblast Giant Cell Development. Edited by Lo CW Tuan RS. Developmental Biology Protocols, Vol III ed. Vol. 137, Methods in Molecular Biology. Totowa, NJ, USA: Humana Press Inc., 2000.

Piccinni MP, Beloni L, Livi C, Maggi E, Scarselli G, Romagnani S. "Defective production of both leukemia inhibitory factor and type 2 T-helper cytokines by decidual T cells in unexplained recurrent abortions." Nat Med. 4, *no.9* (1998):1020-4.

Pileggi A, Molano RD, Berney T, Cattan P, Vizzardelli C, Oliver R, Fraker C, Ricordi C, Pastori RL, Bach FH, Inverardi L. "Heme Oxygenase-1 Induction in Islet Cells Results in Protection from Apoptosis and Improved in Vivo Function after Transplantation." Diabetes. 50, *no. 9* (2001): 1983-91.

Poss KD, Thomas MJ, Ebralidze AK, O´Dell TJ, Tonegawa S. "Hippocampal long-term potentiation is normal in heme oxygenase-2 mutant mice." Neuron 15 (1995): 867-73.

Poss K, Tonegawa S. "Reduced Stress Defense in Heme Oxygenase-1 Deficient Cells." Proc Natl Acad Sci USA. 94 (1997): 10925-30.

Psychoyos A. "Hormonal Control of Uterine Receptivity for Nidation." J Reprod Fertil Suppl. 25 (1976): 17-28.

Psychoyos A. "Uterine Receptivity for Nidation." Ann N Y Acad Sci. 476 (1986): 36-42.

Raghupathy R. "Th1-Type Immunity Is Incompatible with Successful Pregnancy." Immunol Today. 18 (1997): 478-82.

Raghupathy R, Makhseed M, Azizieh F, Omu A, Gupta M, Farhat R. "Cytokine production by maternal lymphocytes during normal human pregnancy and in unexplained recurrent spontaneous abortion." Hum Reprod. 15, *no. 3* (2000): 713-8.

Redaelli CA, Tian YH, Schaffner T, Ledermann M, Baer HU, Dufour JF. "Extended preservation of rat liver graft by induction of heme oxygenase-1." Hepatology 35, *no. 5* (2002): 1082-92.

Redline RW. "Role of Uterine Natural Killer Cells and Interferon Gamma in Placental Development." J Exp Med. 192, *no. 2* (2000): F1-4.

Ririe KM, Rasmussen RP, Wittwer CT. "Product differentiation by analysis of DNA melting curves during the polymerase chain reaction." Anal Bioch. 245 (1997): 154-160.

Ritter T, Vogt K, Rieck P, Schilling-Schon A, Kolls J, Hartmann C, Volk HD, Pleyer U. "Adenovirus-Mediated Gene Transfer of Interleukin-4 to Corneal Endothelial Cells and Organ Cultured Corneas Leads to High Il-4 Expression." Exp Eye Res. 69 (1999): 563-68.

Ryter SW, Tyrrell RM. "The Heme Synthesis and Degradation Pathways: Role in Oxidant Sensitivity. Heme Oxygenase Has Both Pro- and Antioxidant Properties." Free Radic Biol Med. 28, *no. 2* (2000): 289-309.

Ryter SW, Otterbein LE, Morse D, Choi AM. "Heme Oxygenase/Carbon Monoxide Signaling Pathways: Regulation and Functional Significance." Mol Cell Biochem 234-235, *no. 1-2* (2002): 249-63.

Ryter SW, Otterbein LE. "Carbon Monoxide in Biology and Medicine." Bioessays. 26, *no. 3* (2004): 270-80.

Ryter SW, Alam J, Choi AM. "Heme Oxygenase-1/Carbon Monoxide: From Basic Science to Therapeutic Applications." Physiol Rev 86, *no. 2* (2006): 583-650.

Sahgal N, Canham LN, Konno T, Wolfe MW, Soares MJ. "Modulation of trophoblast stem cell and giant cell phenotypes: analyses using the Rcho-1 cell model." Differentiation 73 (2005): 452-62.

Saito S. "Cytokine Network at the Feto-Maternal Interface." J Reprod Immunol. 47, *no. 2* (2000): 87-103.

Saito S, Sasaki Y, Sakai M. "CD4+CD25high regulatory T cells in human pregnancies." J Reprod Immunol. 65, *no. 2* (2005): 111-20.

Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. "Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases." J Immunol. 155, *no. 3* (1995): 1151-64.

Sarady JK, Otterbein SL, Liu F, Otterbein LE, Choi AM. "Carbon monoxide modulates endotoxin-induced production of granulocyte macrophage colony-stimulating factor in macrophages." Am. J. Respir. Cell Mol. Biol. 27 (2002): 739–745

Sasaki Y, Sakai M, Miyazaki S, Higuma S, Shiozaki A, Saito S. "Decidual and peripheral blood CD4+CD25+ regulatory T cells in early pregnancy subjects and spontaneous abortion cases." Mol Hum Reprod. 10, *no. 5* (2004): 347-53.

Sass G, Soares MC, Yamashita K, Seyfried S, Zimmermann WH, Eschenhagen T, Kaczmarek E, Ritter T, Volk HD, Tiegs G. "Heme Oxygenase-1 and Its Reaction Product, Carbon Monoxide, Prevent Inflammation-Related Apoptotic Liver Damage in Mice." Hepatology. 38, *no. 4* (2003): 909-18.

Sato K, Balla J, Otterbein L, Smith RN, Brouard S, Lin Y, Csizmadia E, Sevigny J, Robson SC, Vercellotti G, Choi AM, Bach FH, Soares MP. "Carbon monoxide generated by heme oxygenase-1 suppresses the rejection of mouse-to-rat cardiac transplants." J. Immunol. 166 (2001):4185–94.

Schachtner S, Buck C, Bergelson J, Baldwin H. "Temporally Regulated Expression Patterns Following in Utero Adenovirus-Mediated Gene Transfer." Gene Ther. 6, *no. 7* (1999): 1249-57.

Senoo M, Matsubara Y, Fujii K, Nagasaki Y, Hiratsuka M, Kure S, Uehara S, Okamura K, Yajima A, Narisawa K. "Adenovirus-Mediated in Utero Gene Transfer in Mice and Guinea Pigs: Tissue Distribution of Recombinant Adenovirus Determined by Quantitative Taqman-Polymerase Chain Reaction Assay." Mol Genet Metab. 69, *no. 4* (2000): 269-76.

Senturk LM, Arici A. Leukemia Inhibitory Factor in Reproduction. Edited by Mor G, Immunology of Pregnancy. Georgetown, Texas, USA: Landes Bioscience/Eurekah.com, 2006.

Sepiashvili RI, Shubich MG, Kolesnikova NV, Slavyanskaya TA, Lomtatidze LV. "Apoptosis in Immunologic Processes." Clin Applied Immunol Rev. 1, *no. 3-4* (2001): 163-72.

Sharkey A. "Cytokines and Implantation." Rev Reprod. 3, *no. 1* (1998): 52-61.

Shibahara S, Müller R, Taguchi H, Yoshida T. "Cloning and Expression of Cdna for Rat Heme Oxygenase." Proc Natl Acad Sci U S A. 82, *no. 23* (1985): 7865-69.

Slebos DJ, Ryter SW, van der Toorn M, Liu F, Guo F, Baty CJ, Karlson JM, Watkins SC, Kim HP, Wang X, Lee JS, Postma DS, Kauffman HF, Choi AM. "Mitochondrial localization and function of heme oxygenase-1 in cigarette smoke-induced cell death." Am J Respir Cell Mol Biol. 36, *no. 4* (2007): 409-17.

Smith TA, Mehaffey MG, Kayda DB, Saunders JM, Yei S, Trapnell BC, McClelland A, Kaleko M. "Adenovirus mediated expression of therapeutic plasma levels of human factor IX in mice." Nat Genet. 5*, no. 4* (1993): 397-402.

Smythe JA, Sun D, Thomson M, Markham PD, Reitz MS Jr, Gallo RC, Lisziewicz J. "A Rev-Inducible Mutant Gag Gene Stably Transferred into T Lymphocytes: An Approach to Gene Therapy against Human Immunodeficiency Virus Type 1 Infection." Proc Natl Acad Sci U S A 91, *no. 9* (1994): 3657-61.

Soares MP, Lin Y, Anrather J, Csizmadia E, Takigami K, Sato K, Grey ST, Colvin RB, Choi AM, Poss KD, Bach FH. "Expression of heme oxygenase-1 can determine cardiac xenograft survival." Nat Med. 4, *no. 9* (1998): 1073-7.

Soares MP, Brouard S, Smith RN, Bach FH. "Heme Oxygenase-1, a Protective Gene That Prevents the Rejection of Transplanted Organs." Immunol Rev. 184 (2001): 275-85.

Sollwedel A, Bertoja AZ, Zenclussen ML, Gerlof K, Lisewski U, Wafula P, Sawitzki B, Woiciechowsky C, Volk HD, Zenclussen AC. "Protection from Abortion by Heme Oxygenase-1 up-Regulation Is Associated with Increased Levels of Bag-1 and Neuropilin-1 at the Fetal-Maternal Interface." J Immunol. 175, *no. 8* (2005): 4875-85.

Somia N, Verma IM. "Gene Therapy: Trials and Tribulations." Nat Rev Genet. 1*, no. 2* (2000): 9-19.

Song R, Ning W, Liu F, Ameredes BT, Calhoun WJ, Otterbein LE, Choi AM. "Regulation of IL-1beta-induced GM-CSF production in human airway smooth muscle cells by carbon monoxide" Am. J. Physiol. Lung Cell. Mol. Physiol. 284 (2003): L50–6.

Steck T, Giess R, Suetterlin MW, Bolland M, Wiest S, Poehls UG, Dietl J. "Leukaemia Inhibitory Factor (Lif) Gene Mutations in Women with Unexplained Infertility and Recurrent Failure of Implantation after Ivf and Embryo Transfer." Eur J Obstet Gynecol Reprod Biol. 112, *no. 1* (2004): 69-73.

Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Köntgen F, Abbondanzo SJ. "Blastocyst Implantation Depends on Maternal Expression of Leukaemia Inhibitory Factor." Nature 359, *no. 6390* (1992): 76-79.

Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. "Bilirubin Is an Antioxidant of Possible Physiological Importance." Science. 235, *no. 4792* (1987): 1043-46.

Stocker R, Peterhans E. "Antioxidant Properties of Conjugated Bilirubin and Biliverdin: Biologically Relevant Scavenging of Hypochlorous Acid." Free Radic Res Commun. 6, *no. 1* (1989): 57-66.

Stocker R. "Induction of Haem Oxygenase as a Defence against Oxidative Stress." Free Radic Res Commun 9, *no. 2* (1990): 101-12.

Suttner DM, Dennery PA. "Reversal of HO-1 related cytoprotection with increased expression is due to reactive iron." FASEB J. 13 (1999): 1800–9.

Suzuki T, Hiromatsu K, Ando Y, Okamoto T, Tomoda Y, Yoshikai Y. "Regulatory Role of Gamma Delta T Cells in Uterine Intraepithelial Lymphocytes in Maternal Antifetal Immune Response." J Immunol. 154, *no. 9* (1995): 4476-84.

Svensson L, Arvola M, Sallstrom MA, Holmdahl R, Mattsson R. "The Th2 cytokines IL-4 and IL-10 are not crucial for the completion of allogeneic pregnancy in mice." J Reprod Immunol 51 (2001):3–7.

Szekeres-Bartho J, Barakonyi A, Miko E, Polgar B, Palkovics T. "The Role of Gamma/Delta T Cells in the Feto-Maternal Relationship." Semin Immunol. 13, *no. 4* (2001): 229-33.

Tabibzadeh S, Babaknia A. "The Signals and Molecular Pathways Involved in Implantation, a Symbiotic Interaction between Blastocyst and Endometrium Involving Adhesion and Tissue Invasion." Hum Reprod 10, *no. 6* (1995): 1576-602.

Tafuri A, Alferink J, Möller P, Hämmerling GJ, Arnold B. "T cell awareness of paternal alloantigens during pregnancy." Science 270, *no. 5236* (1995): 630-3.

Takayama S, Sato T, Krajewski S, Kochel K, Irie S, Millan JA, Reed JC. "Cloning and functional analysis of BAG-1: a novel Bcl-2-binding protein with anti-cell death activity." Cell. 80, *no. 2* (1995):279-84.

Tappel AL. "The Mechanism of the Oxidation of Unsaturated Fatty Acids Catalyzed by Hematin Compounds." Arch Biochem Biophys. 44, *no. 2* (1953): 378-95.

Tenhunen R, Marver HS, Schmid R. "The Enzymatic Conversion of Heme to Bilirubin by Microsomal Heme Oxygenase." Proc Natl Acad Sci U S A 61, *no. 2* (1968): 748-55.

Tenhunen R., Marver H.S., Schmid R. "Microsomal Heme Oxygenase- Characterization of the Enzyme." The Journal of Biological Chemistry. 244, *no. 23* (1969): 6388-94.

Tenhunen R, Marver H, Pimstone NR, Trager WF, Cooper DY, Schmid R. "Enzymatic Degradation of Heme. Oxygenative Cleavage Requiring Cytochrome P-450." Biochemistry. 11, *no. 9* (1972): 1716-20.

Thellin O, Coumans B, Zorzi W, Igout A, Heinen E. "Tolerance to the Foeto-Placental 'Graft': Ten Ways to Support a Child for Nine Months." Curr Opin Immunol 12, *no. 6* (2000): 731-37.

Themis M, Schneider H, Kiserud T, Cook T, Adebakin S, Jezzard S, Forbes S, Hanson M, Pavirani A, Rodeck C, Coutelle C. "Successful Expression of Beta-Galactosidase and Factor Ix Transgenes in Fetal and Neonatal Sheep after Ultrasound-Guided Percutaneous Adenovirus Vector Administration into the Umbilical Vein." Gene Ther. 6, *no. 7* (1999): 1200-01.

Thyagarajan B, Phillips JE, Calos MP. Gene Delivery by Viruses, Encyclopedia of Life Sciences. Chichester, UK: John Wiley & Sons Ltd., 2001.

Townsend P, Cutress A, Sharp RI, Brimmell A, Packham MG. "BAG-1: a multifunctional regulator of cell growth and survival" Biochim. Biophys. Acta 1603 (2003): 83–98.

Townsend PA, Cutress RI, Carroll CJ, Lawrence KM, Scarabelli TM, Packham G, Stephanou A, Latchman DS. "BAG-1 proteins protect cardiac myocytes from simulated ischemia/reperfusion-induced apoptosis via an alternate mechanism of cell survival independent of the proteasome." J. Biol. Chem. 279 (2004): 20723–78.

Trakshel GM, Kutty RK, Maines MD. "Purification and Characterization of the Major Constitutive Form of Testicular Heme Oxygenase. The Noninducible Isoform." J Biol Chem. 261, *no. 24* (1986): 11131-37.

Tsokos GC, Goust J-M, Virella G. Tolerance and Autoimmunity. Edited by Virella G. Fifth Edition ed, Medical Immunology. New York, USA: Marcel Dekker, Inc. New York, 2001.

Tullius SG, Nieminen-Kelha M, Buelow R, Reutzel-Selke A, Martins PN, Pratschke J, Bachmann U, Lehmann M, Southard D, Iyer S, Schmidbauer G, Sawitzki B, Reinke P, Neuhaus P, Volk HD. "Inhibition of ischemia/reperfusion injury and chronic graft deterioration by a single-donor treatment with cobalt-protoporphyrin for the induction of heme oxygenase-1." Transplantation 74, *no. 5* (2002): 591-8.

Tyrrell R. "Redox regulation and oxidant activation of heme oxygenase-1." Free Radic. Res. 31 (1999): 335–40.

Vachharajani TJ, Work J, Issekutz AC, Granger DN. "Heme Oxygenase Modulates Selectin Expression in Different Regional Vascular Beds." Am J Physiol Heart Circ Physiol 278, *no. 5* (2000): H1613-17.

Van der Welden AW, Thomas AAM. "The role of the 5´untranslated region of an mRNA in translation regulation during development." Int J Biochem Cell Biol. 31 (1999): 87-106.

Venturini CM, Isakson P, Needleman P. "Non-steroidal anti-inflammatory drug-induced renal failure: a brief review of the role of cyclo-oxygenase isoforms." Curr Opin Nephrol Hypertens. 7, *no. 1* (1998): 79-82.

Vercellotti GM, Balla G, Balla J, Nath K, Eaton JW, Jacob HS "Heme and the vasculature: an oxidative hazard that induces antioxidant defenses in the endothelium. Artif. Cells Blood Substit. Immobil. Biotechnol. 22 (1994): 207–213.

Wagener FA, Feldman E, de Witte T, Abraham NG. "Heme Induces the Expression of Adhesion Molecules Icam-1, Vcam-1, and E Selectin in Vascular Endothelial Cells." Proc Soc Exp Biol Med. 216, *no. 3* (1997): 456-63.

Wagener FA, Volk HD, Willis D, Abraham NG, Soares MP, Adema GJ, Figdor CG. "Different Faces of the Heme-Heme Oxygenase System in Inflammation." Pharmacol Rev. 55, *no. 3* (2003): 551-71.

Waldmann H, Cobbold S. "Regulating the Immune Response to Transplants. A Role for Cd4+ Regulatory Cells?" Immunity 14, *no. 4* (2001): 399-406.

Waldmann H, Cobbold SP, Fairchild P, Adams E. "Therapeutic Aspects of Tolerance." Curr Opin Pharmacol. 1, *no. 4* (2001): 392-97.

Walsh CE. "Fetal Gene Therapy." Gene Ther. 6, *no. 7* (1999): 1200-01.

Walther W, Stein W. "Cell type specific and inducible promoters for vectors in gene therapy as an approach for cell targeting." J Mol Med. 74 (1996): 379-92.

Wang H, Dey SK. "Roadmap to Embryo Implantation: Clues from Mouse Models." Nat Rev Genet. 7, *no. 3* (2006): 185-99.

Weber CM, Eke BC, Maines MD. "Corticosterone Regulates Heme Oxygenase-2 and No Synthase Transcription and Protein Expression in Rat Brain." J Neurochem. 63, *no. 3* (1994): 953-62.

Wegmann TG, Lin H, Guilbert L, Mosmann TR. "Bidirectional Cytokine Interactions in the Maternal-Fetal Relationship: Is Successful Pregnancy a Th2 Phenomenon?" Immunol Today 14, *no. 7* (1993): 353-56.

Willis D, Moore AR, Frederick R, Willoughby DA. "Heme Oxygenase: A Novel Target for the Modulation of the Inflammatory Response." Nat Med. 2, *no. 1* (1996): 87-90.

Wilson JM. "Adenoviruses as gene-delivery vehicles." N Engl J Med 334 (1996): 1185-7.

Wise CD, Drabkin DL. "Degradation of Haemoglobin and Hemin to Biliverdin by a New Cell-Free System Obtained from the Hemophagous Organ of Dog Placenta." Fed. Proc. 23 (1964): 323.

Woo J, Iyer S, Mori N, Buelow R. "Alleviation of graft-versus-host disease after conditioning with cobalt-protoporphyrin, an inducer of heme oxygenase-1." Transplantation 69, *no. 4* (2000): 623-33.

Wood KJ, Fry J. "Gene Therapy: Potential Applications in Clinical Transplantation." Expert Rev Mol Med. 1999 (1999): 1-20.

Wood KJ, Prior TGT. "Gene Therapy in Transplantation." Curr Opin Molecular Therapeutics 3, *no. 4* (2001): 390-98.

Wood KJ, Sakaguchi S. "Regulatory T Cells in Transplantation Tolerance." Nat Rev Immunol 3, *no. 3* (2003): 199-210.

Wu M, Ding H-F, Fisher DE. Apoptosis: Molecular Mechanisms, Encyclopedia of Life Sciences. Chichester, UK: John Wiley & Sons Ltd., 2001.

Yachie A, Niida Y, Wada T, Igarashi N, Kaneda H, Toma T, Ohta K, Kasahara Y, Koizumi S. "Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency." J Clin Invest. 103, *no. 1* (1999): 129-35.

Yamashita K, McDaid J, Ollinger R, Tsui TY, Berberat PO, Usheva A, Csizmadia E, Smith RN, Soares MP, Bach FH. "Biliverdin, a Natural Product of Heme Catabolism, Induces Tolerance to Cardiac Allografts." FASEB J. 18, *no. 6* (2004): 765-67.

Yamashita K, Ollinger R, McDaid J, Sakahama H, Wang H, Tyaqi S, Csizmadia E, Smith NR, Soares MP, Bach FH. "Heme oxygenase-1 is essential for and promotes tolerance to transplanted organs." FASEB J. 20, *no. 6* (2006): 776-8.

Yang J, Ajonuma LC, Rowlands DK, Tsang LL, Ho LS, Lam SY, Chen WY, Zhou CX, Chung YW, Cho CY, Tse JY, James AE, Chan HC. "The role of inducible nitric oxide synthase in gamete interaction and fertilization: a comparative study on knockout mice of three NOS isoforms." Cell Biol Int. 29 (2005): 785-91.

Yet SF, Perrella MA, Layne MD, Hsieh CM, Maemura K, Kobzik L, Wiesel P, Christou H, Kourembanas S, Lee ME. "Hypoxia Induces Severe Right Ventricular Dilatation and Infarction in Heme Oxygenase-1 Null Mice." J Clin Invest. 103, *no. 8* (1999): R23-29.

Yoshida T, Kikuchi G. "Sequence of the Reaction of Heme Catabolism Catalyzed by the Microsomal Heme Oxygenase System." FEBS Lett. 48, *no. 2* (1974): 256-61.

Zelenay S, Chora A, Soares MP, Demengeot J. "Heme oxygenase-1 is not required for mouse regulatory T cell development and function." Int Immunol. 19, *no. 1* (2007): 11-8.

Zenclussen AC, Kortebani G, Mazzolli A, Margni R, Malan Borel I. "Interleukin-6 and Soluble Interleukin-6 Receptor Serum Levels in Recurrent Spontaneous Abortion Women Immunized with Paternal White Cells." Am J Reprod Immunol 44, *no. 1* (2000): 22-29.

Zenclussen AC, Fest S, Sehmsdorf US, Hagen E, Klapp BF, Arck PC. "Upregulation of Decidual P-Selectin Expression Is Associated with an Increased Number of Th1 Cell Populations in Patients Suffering from Spontaneous Abortions." Cell Immunol. 213, *no. 2* (2001): 94-103.

Zenclussen AC, Fest S, Busse P, Joachim R, Klapp BF, Arck PC. "Questioning the Th1/Th2 paradigm in reproduction: peripheral levels of IL-12 are down-regulated in miscarriage patients." Am J Reprod Immunol. 48, *no. 4* (2002): 245-51.

Zenclussen AC, Joachim R, Hagen E, Peiser C, Klapp BF, Arck PC. "Heme Oxygenase Is Downregulated in Stress-Triggered and Interleukin-12-Mediated Murine Abortion." Scand. J. Immunol. 55 (2002): 560-69.

Zenclussen AC, Blois S, Stumpo R, Olmos S, Arias K, Malan Borel I, Roux ME, Margni RA. "Murine Abortion Is Associated with Enhanced Interleukin-6 Levels at the Feto-Maternal Interface." Cytokine. 24 (2003): 150-60.

Zenclussen AC, Lim E, Knoeller S, Knackstedt M, Hertwig K, Hagen E, Klapp BF, Arck PC. "Heme Oxygenases in Pregnancy II: HO-2 Is Downregulated in Human Pathologic Pregnancies." Am J Reprod Immunol. 50 (2003): 66-76.

Zenclussen AC, Fest S, Joachim R, Klapp BF, Arck PC. "Introducing a Mouse Model for Pre-Eclampsia: Adoptive Transfer of Activated Th1 Cells Leads to Pre-Eclampsia-Like Symptoms Exclusively in Pregnant Mice." Eur J Immunol. 34 (2004): 377-87.

Zenclussen AC. "CD4+ CD25+ T regulatory cells in murine pregnancy." J Reprod Immunol. 65, *no. 2* (2005): 101-10.

Zenclussen AC, Gerlof K, Zenclussen ML, Sollwedel A, Bertoja AZ, Ritter T, Kotsch K, Leber J, Volk HD. "Abnormal T-Cell Reactivity against Paternal Antigens in Spontaneous Abortion: Adoptive Transfer of Pregnancy-Induced Cd4+Cd25+ T Regulatory Cells Prevents Fetal Rejection in a Murine Abortion Model." Am J Pathol. 166, *no. 3* (2005): 811-22.

Zenclussen AC, Sollwedel A, Zambon Bertoja A, Gerlof K, Zenclussen ML, Woiciechowsky C, Volk HD. "Heme Oxygenase as a Therapeutic Target in Immunological Pregnancy Complications." Int Immunopharmacol. 5, *no. 1* (2005): 41-51.

Zenclussen AC, Gerlof K, Zenclussen ML, Ritschel S, Zambon Bertoja A, Fest S, Hontsu S, Ueha S, Matsushima K, Leber J, Volk HD. "Regulatory T Cells Induce a Privileged Tolerant Microenvironment at the Fetal-Maternal Interface." Eur J Immunol. 36, *no. 1* (2006): 82-94.

Zenclussen ML, Anegon I, Bertoja AZ, Chauveau C, Vogt K, Gerlof K, Sollwedel A, Volk HD, Ritter T, Zenclussen AC. "Over-expression of heme oxygenase-1 by adenoviral gene transfer improves pregnancy outcome in a murine model of abortion." J Reprod Immunol. 69, *no. 1* (2006): 35-52.

Zygmunt M, Herr F, Münstedt K, Lang U, Liang OD. "Angiogenesis and vasculogenesis in pregnancy." Eur J Obstet Gynecol Reprod Biol. 110, *no. 1* (2003): S10-8.

Appendix

Abortion rates using high doses of adenoviral vectors:

Table 14 : The application of a higher dose of AdHO-1/GFP did not significantly change abortion rates.

	N.P	*S.A.*	*S.A. + 1.10^{8}PFU AdHO-1/GFP*	S.A. + *1.10^8 PFU* AdEGFP
Mean	2.61	24.73	19.28	22.16
SD	5.42	7.06	10.37	12.86
Median	0	25***	19.4	25

Mean, SD and median of abortion rates. N.P.= Normal Pregnancy; S.A.= Spontaneous abortion.***$p \leq 0.001$ when compared to NP by the Mann-Whitney test

Ex-vivo cytokine production as measured by flow cytometry:

Table 15 : *ex-vivo* cytokine production by decidual lymphocytes

	IL-4	IL-10	IFN-γ	TNF-α
N.P.	3.63	2.73	3.96	9.21
S.A.	6.75	7.58	10.47	10.66
S.A. + 1.10^5 AdHO-1/GFP	13.10	2.76	3.83	23.07
S.A. + 1.10^5 AdEGFP	1.00	2.30	4.00	6.38
S.A. + 1.10^8 AdHO-1/GFP	9.69	14.68	17.34	18.65
S.A. + 1.10^8 AdHO-1/GFP	6.05	2.63	2.69	19.89

Cytokine production measured by flow cytometry after PMA/Ionomycin incubation in the presence of monensin. Data are showed as medians. N.P.= Normal Pregnancy; S.A.= Spontaneous abortion.

Table 16 : *ex-vivo* cytokine production by spleen lymphocytes

	IL-4	***IL-10***	***IFN-γ***	***TNF-α***
N.P.	1.15	0.95	1.08	2.28
S.A.	0.65	1.00	1.20	6.54
S.A. + 1.10^5 AdHO-1/GFP	2.57	1.42	1.13	2.57
S.A. + 1.10^5 AdEGFP	0.45	0.38	0.63	1.10
S.A. + 1.10^8 AdHO-1/GFP	0.45	0.63	0.60	6.41
S.A. + 1.10^8 AdHO-1/GFP	1.45	1.57	1.59	2.58

Cytokine production measured by flow cytometry after PMA/Ionomycin incubation in the presence of monensin. Data are showed as medians. N.P.= Normal Pregnancy; S.A.= Spontaneous abortion.

Levels of HO-1 mRNA in the groups receiving high doses of adenovirus:

Table 17 : mRNA levels of HO-1 normalized to β-actin

	N.P	***S.A.***	***S.A.* + *1.10^{8}PFU AdHO-1/GFP***	**S.A. + *1.10^8 PFU* AdEGFP**
Mean	0,05076	0,03590	0,06676	0,04263
SD	0,04651	0,01383	0,01629	0,01912
Median	0,02906	0,02967	0,06846*	0,03584

N.P.= Normal Pregnancy; S.A.= Spontaneous abortion. *p≤0,05 as analyzed by the Mann-Whitney U-test for two particular groups. A significant difference was obtained in the group receiving 1.10^8 PFU AdHO-1/GFP when compared to the S.A. group.

Curriculum Vitae

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Publikationsliste

Wissenschaftliche Veröffentlichungen:

Amadeo I., Oggero M., **Zenclussen M.L**., Robles L., Pereira D., Kratje R., Etcheverrigaray M.: A single monoclonal antibody as probe to detect the entire set of native and partially unfolded rhEPO glycoforms. J Immunol Methods. 2004; *293(1-2*): 191-205.

Zenclussen A.C., Sollwedel A., Zambon Bertoja A., Gerlof K., **Zenclussen M.L**., Woiciechowsky C., Volk H.D.: Heme Oxygenase as a therapeutic target in immunological pregnancy complications. Int Immunopharmacol. 2005*; 5(1)*:41-51.

Zenclussen A.C., Gerlof K., **Zenclussen M.L**., Sollwedel A., Zambon Bertoja A., Ritter T., Kotsch K., Leber J., Volk H.D.: Abnormal T cell reactivity against paternal antigens in spontaneous abortion: Adoptive transfer of pregnancy-induced CD4+CD25+ T regulatory cells prevents fetal rejection in a murine abortion model. Am J Pathol. 2005; *166(3):*811-22.

Zambon Bertoja A., **Zenclussen M.L**., Wollenberg I., Paeschke S., Sollwedel A., Gerlof K., Woiciechowsky C., Volk H.D., Zenclussen A.C.: Upregulation of bcl-2 at the foetal-maternal interface from mice undergoing abortion. Scand J Immunol. 2005; *61(6):*492-502.

Sollwedel A, Bertoja AZ, **Zenclussen M.L.**, Gerlof K, Lisewski U, Wafula P, Sawitzki B, Woiciechowsky C, Volk HD, Zenclussen AC.: Protection from abortion by heme oxygenase-1 up-regulation is associated with increased levels of Bag-1 and neuropilin-1 at the fetal-maternal interface. J Immunol.2005; *175(8*):4875-85.

Zenclussen A.C., **Zenclussen M.L**., Ritter T., Volk H.D.: The use of gene therapy tools in reproductive immunology research. Curr Gene Therapy 2005; *5*: 459-466.

Paeschke S., Chen F., Horn N., Fotopoulou C., Zambon-Bertoja A., Sollwedel A., **Zenclussen M.L.**, Casalis P.A., Dudenhausen J.W., Volk H.D., Zenclussen A.C.: Pre-eclampsia is not associated with changes in the levels of regulatory T cells in peripheral blood. Am J Reprod Immunol 2005; *54(6):*384-9.

Zenclussen A.C., Gerlof K., **Zenclussen M.L**., Ritschel S., Zambon Bertoja A., Fest S., Hontsu S., Ueha S., Matsushima K., Leber J., Volk H.D.: Regulatory T cells induce a privileged tolerant microenvironment at the fetal-maternal interface. Eur J Immunol. 2006; *36(1*):82-94.

Zenclussen M.L., Anegon I., Bertoja A.Z., Chauveau C., Vogt K., Gerlof K., Sollwedel A., Volk H.D., Ritter T., Zenclussen A.C. : Over-expression of heme oxygenase-1 by adenoviral gene transfer improves pregnancy outcome in a murine model of abortion. J Reprod Immunol. 2006; *69(1):*35-52.

Bertoja A.Z., **Zenclussen M.L.**, Casalis P.A., Sollwedel A., Schumacher A., Woiciechowsky C., Volk H.D., Zenclussen A.C.: Anti-P- and E-selectin therapy prevents abortion in the CBA/J x DBA/2J combination by blocking the migration of Th1 lymphocytes into the foetal-maternal interface. Cell Immunol. 2005; *238(2*):97-102.

Schmid M., Sollwedel A., Thuere C., Wafula P.O., **Zenclussen M.L**., Müller D.N., Gratze P., Woiciechowsky C., Volk H.D., Zenclussen A.C.: Murine pre-eclampsia induced by unspecific activation of the immune system correlates with alterations in the eNOS and AT1 receptor expression in the kidneys and placenta. Placenta 2007; *28(7):*688-700.

Zenclussen A.C., Schumacher A., **Zenclussen M.L**., Wafula P., Volk H.D.: Immunology of pregnancy: cellular mechanisms allowing fetal survival within the maternal uterus. Expert Rev Mol Med. 2007; *9(10*):1-14.

Thuere C., **Zenclussen M.L**., Schumacher A., Langwisch S., Schulte-Wrede U., Teles A., Paeschke S., Volk H.D., Zenclussen A.C.: Kinetics of Regulatory T cells during murine pregnancy. Am J Reprod Immunol. 2007; *58*: 514-523.

Fest S., Brachwitz N., Schumacher A., **Zenclussen M.L.,** Khan F., Wafula P.O., Casalis P.A., Fill S., Costa S.D., Mor G., Volk H.D., Lode H.N., Zenclussen A.C. Supporting the hypothesis of pregnancy as a tumor: survivin is upregulated in normal pregnant mice and participates in human trophoblast proliferation. Am J Reprod Immunol. 2008; *59(1):*75-83.

Zenclussen M.L., Casalis P.A., Volk H.D., Zenclussen A.C. Heme Oxygenase-1 (HO-1) is essential for trophoblast survival and differentiation. In Vorbereitung

Abstracts:

Zenclussen M.L., Oggero Eberhardt M, Kratje M, Etcheverrigaray M. Revista Medicina Vol. 61 *Nro. 5/2* (2001).

Zenclussen M.L., Oggero Eberhardt M., Kratje R., Etcheverrygaray M: Kinetic and Metabolism Studies of different Hybridomas Producing anti rhEPO Monoclonal Antibodies. ENPROMER III. Biotechnology Session (2002).

Amadeo I., **Zenclussen M.L**., Oggero Eberhardt M., Kratje R., Etcheverrigaray M., Cuantificación de rhEPO mediante un ELISA Sandwich en diferentes estadios del proceso productivo. Colecciones: Química Analítica. Biblioteca Pública Digital Argentina (2003).

Zenclussen M.L., Hempel M., Vogt K., Volk H.D., Ritter T.: Generation of allospecific T-cells expressing HO-1 for its application in transplantation. 2003 Basic Sciences Symposium: Transplantation in the Real World.

Zenclussen M.L., Hempel M., Vogt K., Ecke I., Zenclussen A.C., Volk H.D., Ritter T.: Generation of allospecific Th2-cells expressing Heme Oxygenase-1 (HO-1) for its application in transplantation. Immunobiology, Volume 208, *Issues 1-3*, (2003).

Zenclussen M.L., Ritter T., Volk H.D., Zenclussen, A.C.: Upregulation of Heme Oxygenase-1 (HO-1) through adenoviral gene transfer improves pregnancy outcome in a murine model of abortion. Immunobiology, Volume 208, *Issues 1-3*, (2003).

Zambon Bertoja AR, Paeschke S., **Zenclussen M.L**., Sollwedel A., Leber J., Ritter T., Volk H.D., Zenclussen A.C.: Pro-and anti-apoptotic molecules expression during murine pregnancy. Immunobiology, Volume 208, *Issues 1-3* (2003).

Zenclussen A.C., Zambon Bertoja A., Sollwedel A., Leber J., **Zenclussen M.L**., Paeschke S., Woiciechowsky C., Volk H.D.: Ex-vivo production of Th1 and Th2 cytokines as well as chemokine receptors expression by decidual and placental cells in an immunological abortion mouse model. Immunobiology, Volume 208, *Issues 1-3* (2003).

Zenclussen M.L., Anegon I., Zambon Bertoja A., Vogt K., Sollwedel A., Gerlof K., Ziemert N., Volk H.-D., Ritter T., Zenclussen A.C. Systemic application of an adenoviral vector containing the protective molecule heme oxygenase-1 (HO-1) avoids fetal rejection in a murine model. Am J Reprod Immunol 52, *Suppl. 1* (2004): 29.

Zambon Bertoja A., **Zenclussen M.L**., Wollenberg I., Sollwedel A., Paeschke S., Gerlof K., Volk H.-D., Zenclussen A.C. No signs of increased apoptosis in Th1-mediated murine abortion: involvement of pro- and anti-apoptotic molecules. Am J Reprod Immunol 52, *Suppl. 1* (2004): 25.

Sollwedel A.S., Zambon Bertoja A., **Zenclussen M.L**., Gerlof K., Volk H.-D., Zenclussen A.C. Pharmacological downregulation of heme-oxygenase (HO) by Zinc-protoporphyrin boosts murine abortion. Am J Reprod Immunol 52, *Suppl. 1* (2004): 29.

Zenclussen A.C, Gerlof K., **Zenclussen M.L**., Sollwedel A., Zambon Bertoja A., Leber J., Volk H.-D. Abnormal T cell reactivity against paternal antigens in spontaneous abortion: adoptive transfer of pregnancy-induced CD4+CD25+ T regulatory cells avoids fetal rejection in a murine abortion model. Am J Reprod Immunol 52, *Suppl. 1* (2004): 37.

Zenclussen M.L., Ritter T., Zambon Bertoja A., Gerlof K., Vogt K., Anegon I., Volk H.D., Zenclussen A.C.: Gene therapy employing Heme Oxygenase-1 improves pregnancy outcome in a murine model of abortion. 12th International Congress of Immunology, Montreal, Canada (2004).

Oggero Eberhardt M, Amadeo G, **Zenclussen M.L**., Kratje R, Etcheverrigaray M."A single monoclonal antibody as probe to detect the entire set of native and partially unfolded rhEPO glycoforms". Poster P.I.A.4. – Session 1: Posttranslational processing and product quality. Page 10. Cell Culture Engineering IX – Engineering Conferences International (ECI). Cancún, Mexico (2004).

Zenclussen A.C., Gerlof K., **Zenclussen M.L**., Zambon Bertoja K., Volk H.D.: The adoptive transfer of CD4+CD25+ regulatory cells from normal pregnant mice prevents fetal rejection in a murine abortion model. 12th International Congress of Immunology, Montreal, Canada (2004).

Zambon Bertoja A., **Zenclussen M.L**., Paeschke S., Volk H.D., Zenclussen A.C.: No evidences of increased apoptosis in murine abortion probably due to increased expression of anti-apoptotic proteins at the feto-maternal interface. 12th International Congress of Immunology, Montreal, Canada (2004).

Sollwedel A., Zambon Bertoja A., Gerlof K., **Zenclussen M.L**., Volk H.D., Zenclussen A.C.: Upregulation of Heme-oxygenase by Cobalt-Protoporphyrin Treatment prevents murine abortion. 12th International Congress of Immunology, Montreal, Canada (2004).

Gerlof K., **Zenclussen M.L.,** Sollwedel A., Volk H.D., Zenclussen A.C.: CD4+/CD25+ T regulatory (Treg) cells play an important role in maintaining pregnancy. New Trends in Immunosuppression, Salzburg, Austria (2004).

Zenclussen M.L., Zambon Bertoja A., Gerlof K., Ritschel S., Sollwedel A., Volk H.-D., Zenclussen A.C. During pregnancy, treg cells induce a privileged tolerant microenvironment at the fetal-maternal interface by up-regulating HO-1, TGF-b and LIF expression. Am J Reprod Immunol 54 (2005): 121.

Zenclussen M.L., Sollwedel A., Wollenberg I., Rebelo S., Bertoja A.Z., Volk H.-D., Zenclussen A.C. Heme Oxygenase-1 (HO-1) prevents murine abortion. Am J Reprod Immunol 54 (2005): 131.

Zambon Bertoja A., **Zenclussen M.L**., Sollwedel A., Casalis P., Woiciechowsky C., Volk H.-D., Zenclussen A.C. Therapy with anti-P- and anti-E-selectin monoclonal antibodies (MAbs) significantly reduces the abortion rate in the murine combinationCBA/J x DBA/2J by blocking lymphocyte migration into the fetal-maternal interface. Am J Reprod Immunol 53 (2005): 304.

Leber J., **Zenclussen M.L**., Bertoja A.Z., Sollwedel A., Wollenberg I., Gerlof K., Volk H.D., Zenclussen A.C. Chemokine and chemokine receptor expression at the feto-maternal interface during pregnancy in normal and abortion-prone mice. Am J Reprod Immunol 54 (2005): 130.

Sollwedel A., Zambon Bertoja A**., Zenclussen M.L**., Ritschel S., Wollenberg I., Gerlof K., Volk H.D., Zenclussen A.C. Generation of treg cells in the CBA/J x DBA/2J combination by vaccination with male BALB/c splenocytes rescues from abortion. Am J Reprod Immunol 54 (2005): 120.

Schumacher A., Wollenberg I., Bertoja A.Z., Thüre C., Gerlof K., **Zenclussen M.L**., Sollwedel A., Leber J., Wafula P., Volk H.-D., Zenclussen A.C. Pregnancy-protective Treg are antigen-specific. Am J Reprod Immunol 54 (2005): 147

Paeschke S., Horn N., Fotopoulou C., Zambon Bertoja A., Sollwedel A., **Zenclussen M.L**., Casalis P., Dudenhausen J., Volk H.-D., Chen F., Zenclussen A. Comparable levels of CD4+CD25+CTLA4+ Treg cells in peripheral blood of pre-eclampsia and normal pregnant patients. Am J Reprod Immunol 54 (2005): 120.

Zenclussen M.L., Wafula P., Thuere C., Casalis P., Fest S., Bechmann I., Priller J., Volk H.-D., Zenclussen A.C. Paternal Alloantigens are recognized by the Direct Pathway in Maternal Tissues throughout the Whole Murine Pregnancy Beginning on Day 0. *A*m J Reprod Immunol 56 (2006): 60.

Zenclussen A.C., Thuere C., Leber J., Schumacher A., **Zenclussen M.L**., Wafula P., Volk H.-D. Antigen-specific Treg are generated very early in pregnancy. J Reprod Immunol 71, *no.2* (2006): 149-150.

Wollenberg I., Zambon Bertoja A., Rebelo S., Sollwedel A., **Zenclussen M.L**., Volk H.-D., Soares M., Zenclussen A.C. Paternal HO-1 Deficiency might be responsible for Increased Abortion Rates in the CBA/J x DBA/2J Combination and this can be Rescued by Exposition to Carbon monoxide. Am J Reprod Immunol 56 (2006): 60.

Zenclussen A.C., Wollenberg I., Rebelo S., Zambon Bertoja A., **Zenclussen M.L**., Volk H.-D., Soares M. Heme Oxygenase-1 is Indispensable for Successful Pregnancy. Am J Reprod Immunol 56 (2006): 10.

Schumacher A., Wollenberg I., Zambon Bertoja A., Thüre C., Gerlof K., **Zenclussen M.L**., Sollwedel A., Leber J., Wafula P., Volk H.-D., Zenclussen A.C. Pregnancy-protective Regulatory T Cells act in an Antigen-specific Manner. Am J Reprod Immunol 56 (2006): 11.

Thuere C., Leber J., Schumacher A**., Zenclussen M.L**., Volk H.D., Zenclussen A.C. Increased numbers of FoxP3+ cells in vaginal mucus from normal pregnant mice suggest early antigen-specific tolerance mechanism during pregnancy. Am J Reprod Immunol 55 (2006): 390

Wafula P., Schumacher A., Ritschel S., Sollwedel A., **Zenclussen M.L**., Volk H.D., Zenclussen A.C.: Pregnancy-induced Treg cells act in an antigen-specific manner. 7th International Conference on New Trends in Immunosuppression and Immunotherapy, Berlin.

Zenclussen M.L., Rebelo S., Volk H.D., Soares M., Zenclussen A.C.. Involvement of heme oxygenase-1 (HO-1) in ovarian follicle development and fertility. J Reprod Immunol. 75, *Issue 1* (2007): A15.

Brachwitz N., Ritschel S., **Zenclussen M.L**., Sollwedel A., Volk H.D., Zenclussen A.C. HO-1 up-regulation increases the number of uNK at the fetal–maternal interface. J Reprod Immunol. 75, *Issue 1* (2007): A7-A8.

Zenclussen M.L., Rebelo S, Volk HD, Soares MP, Zenclussen AC: Heme oxygenase-1 (HO-1) may be involved in follicle development and fertility. Am J Reprod Immunol. 57 (2007): 387.

Casalis P.A., Griebenow M., **Zenclussen M.L**., Zenclussen A.C., Volk H.D., Woiciechowsky C., Thomale U.W. Effect of injury severity on the local and systemic cytokine expression in a rat model of traumatic brain injury. 37th annual meeting of the german society of immunology (2007).

Brachwitz N., Zenclussen M.L., Sollwedel A., Ritschel S., Volk H.D., Zenclussen A.C. HO-1 up-regulation increases the number of uNK at the feto-maternal interface. 37th annual meeting of the german society of immunology (2007).

Selbstständigkeitserklärung

Hiermit erkläre ich, Maria Laura Zenclussen, daß ich die Doktorarbeit: Role of Heme Oxygenase-1 in the feto-maternal tolerance selbst verfasst habe und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Maria Laura Zenclussen.